

**INVESTIGATING THE PRESENCE OF MITOCHONDRIAL DNA METHYLATION,
ITS ALTERATION AND ASSOCIATION WITH MITOCHONDRIAL
TRANSCRIPTION AND MITOCHONDRIAL FUNCTION**

By
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ABSTRACT

Mitochondrial proteins are encoded by both the nuclear and mitochondrial genome (mtDNA). Thus, alterations in mtDNA affect mitochondrial function and are involved in a plethora of diseases associated with mitochondrial dysfunction. Although role of nuclear DNA methylation in regulating gene expression is fairly well understood, the very existence of mtDNA methylation has been debated for the past four decades. In spite of the controversies revolving mtDNA methylation, differences in mtDNA methylation have been reported in a number of pathological conditions. However, role of mtDNA methylation in regulating transcription of mtDNA and consequently, mitochondrial function remains unexplored. Besides, whether mtDNA methylation can be altered experimentally has not been investigated. The goal of this dissertation is to establish a method to quantitate mtDNA methylation and evaluate its presence in SH-SY5Y neuroblastoma cells; examine if mtDNA methylation can be altered and if there are any associated changes in mitochondrial transcription and function; and further, determine if mtDNA methylation is altered in a model associated with mitochondrial dysfunction.

In Chapter II, we aimed to investigate the presence of mtDNA methylation in SH-SY5Y neuroblastoma cells at global and site-specific levels. In order to investigate the presence of methylation at global levels in SH-SY5Y mtDNA, we established a direct, sensitive and highly specific method using HPLC-ESI-MS/MS by addressing limitations such as differential or incomplete enzymatic digestion of DNA and also, potential nuclear DNA contamination. Differential enzymatic digestion of DNA was addressed by incorporation of one-step digestion for DNA and using oligonucleotides harboring known amounts of dC and 5m-dC as calibrators. 5m-dC was detected in a linear and proportional manner in SH-SY5Y mtDNA. Around 3% methylation was found in mtDNA obtained from crude mitochondrial isolates, which was

comparable to whole-cell DNA methylation levels. Interestingly, similar percentages of methylation were observed even in mtDNA obtained from gradient-purified mitochondria. Further, purity of mtDNA was characterized, indicating elimination of 99.95 % of nuclear DNA contamination with 115 fold-enrichment of mtDNA in gradient-purified mitochondria. Presence of methylation in SH-SY5Y mtDNA was further substantiated using methylation-specific PCR for a specific site in 12S rRNA. Thus, we established a direct method to quantify global methylation in mtDNA using HPLC-MS/MS and also, reported presence of methylated DNA in purified SH-SY5Y mitochondria.

In Chapter III, we investigated if mtDNA methylation can be altered by experimental interventions and whether altered mtDNA methylation has any concomitant effects on mitochondrial transcription and mitochondrial function. Global mtDNA methylation was assessed by HPLC-ESI-MS/MS and site-specific methylation of 12S rRNA was studied by methylation-specific PCR assay. DNMT1 is known to localize in mitochondria but its role in mtDNA methylation is unknown. We reported that transient over-expression of DNMT1 in SH-SY5Y increased mtDNA methylation, decreased transcription of mtDNA-encoded genes and reduced total and basal-mitochondrial oxygen consumption rates. Likewise, treatment of SH-SY5Y by DNA methylation inhibitor (5-aza-dC) reduced levels of mtDNA methylation, increased transcription of mtDNA-encoded genes and altered mitochondrial oxygen consumption rate. Thus, we found that that mtDNA methylation is an alterable phenomenon, which is affected by DNMT1 levels and 5-aza-dC treatment and is associated with changes in mtDNA transcription and mitochondrial function.

In chapter IV, we investigated the direct role of mtDNA in aging-associated mitochondrial dysfunction using cytoplasmic hybrid cell lines that were created by transferring mitochondria from platelets of healthy young and aged human subjects to mtDNA-depleted SH-SY5Y ρ^0 cells. Bioenergetic fluxes, their regulators and DNA methylation-associated parameters were characterized in these resulting cytoplasmic hybrids. Relative to the cybrids generated from young donors, mitochondrial oxygen consumption parameters and COX activity were reduced in the cybrids made from aged subjects. Interestingly, there was a compensatory increase of Complex I activity in the cybrids generated from aged subjects. However, no alterations were found in ATP levels, redox states and glycolysis fluxes. Although the mitochondrial mass was not different, mRNA levels of some of the mtDNA-encoded OXPHOS genes and nuclear-encoded genes that promote mitochondrial biogenesis were induced in the cybrids created from aged subjects. Although total DNA methylation remained unaltered, levels of DNMT1 protein, mtDNMT1 mRNA and methylation in the mtDNA were higher in the cybrids generated from aged subjects. Thus, we found that mtDNA have a direct role in age-associated mitochondrial dysfunction and mtDNA methylation is altered in age-dependent manner in cybrid model.

In summary, this dissertation work established a method to detect global mtDNA methylation and quantified methylation levels in isolated mitochondria of characterized purity. Also, it established that mtDNA methylation is an alterable phenomenon, which is affected by DNMT1 levels and 5-aza-dC treatment and is associated with mitochondrial transcription and mitochondrial function. Further, it established a direct role of mtDNA in age-associated mitochondrial dysfunction and alteration in mtDNA methylation as one of the age-dependent changes in mtDNA.

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ABBREVIATIONS

2-DG	2-deoxyglucose
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
ATP6	ATP synthase F0 subunit 6
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APOE	Apo lipoprotein E
Bp	Base pair
CNS	Central nervous system
CGI	CpG islands
CSB	Conserved sequence block
COX1	Cytochrome c oxidase subunit 1
COX2	Cytochrome c oxidase subunit 2
COX3	Cytochrome c oxidase subunit 3
CREB	cAMP-response element binding protein
Cybrid	Cytoplasmic hybrid
CytB	Cytochrome B
dA	2'-deoxy-adenosine
dC	2'-deoxy-cytidine
dG	2'-deoxy-guanine
dT	2'-deoxy-thymine
DNMT	DNA methyl transferase
DMEM	Dulbecco's Modified Eagle Medium

ECAR	Extracellular acidification rate
ERR- α	Estrogen-related receptor alpha
ETC	Electron transport chain
ESC	Embryonic stem cells
ELISA	Enzyme-linked Immunosorbent assay
FADH ₂	Flavin adenine dinucleotides
FBS	Fetal bovine serum
HBSS	Hank's Balanced Salt Solution
HDAC	Histone deacetylases
HMT	Histone-methyl-transferases
HPLC-ESI-MS/MS	High Pressure Liquid Chromatography-Electron Spray Ionization- Mass Spectrometry in tandem
HPLC-MS	High Pressure Liquid Chromatography- Mass Spectrometry
HSP	H-strand promoter
5-hmC	5-hydroxymethyl cytosine
5-mdC	5-methyl-2'-deoxy-cytidine
ICF	Immunodeficiency Centromere instability and Facial anomalies syndrome
IRSs	Interspersed repetitive sequences
LSP	L-strand promoter
MBP	Methyl-DNA-binding protein
5-mC	5-methyl-cytosine
Me DIP	Methylated DNA-IP
MEF	Mouse embryonic fibroblast
mRNA	Messenger Ribonucleic Acid
MSRE	Methylation-sensitive restriction enzyme

MtDNA	Mitochondrial Deoxy-ribonucleic Acid
MtDNMT1	Mitochondrial isoform of DNMT1
MSHE	Mannitol-Sucrose-HEPES-EGTA
mtSSB	Mitochondrial single stranded DNA binding protein
NADH	Nicotinamide adenine dinucleotide
NASH	Non-alcoholic steatohepatitis
NCR	Non-coding region
ND1	NADH dehydrogenase subunit 1
ND2	NADH dehydrogenase subunit 2
ND3	NADH dehydrogenase subunit 1
ND4	NADH dehydrogenase subunit 2
ND5	NADH dehydrogenase subunit 1
ND6	NADH dehydrogenase subunit 2
NRF1	Nuclear respiratory factor 1
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PGC-1	Peroxisomal proliferator-activated receptor-gamma co-activator 1
POLRMT	Polymerase RNA of mitochondria
PPAR- γ	Peroxisomal proliferator activated receptor γ
PRC	PGC-1 related co-activator
PSEN1	Presenelin 1

ROS	Reactive oxygen species
ρ^0	Rho-zero cells
rRNA	Ribosomal Ribonucleic Acid
SAH	S-Adenosyl-L-homocysteine
SAM	S-Adenosyl-L-methionine
SIRT1	NAD-dependent deacetylase sirtuin-1
SNCA	α -synuclein
TERMF1	Termination factor 1
TLC	Thin Layer Chromatography
TFB	Transcription factor B
TFAM	Transcription factor A of mitochondria
TNF- α	Tumor necrosis factor alpha
TSS	Transcriptional start sites
tRNA	Transfer Ribonucleic Acid
VEGF-A	Vascular endothelial growth factor A

CHAPTER I: INTRODUCTION

1.1 MITOCHONDRIAL DNA AND ITS FUNCTION

1.1.1 MITOCHONDRIA AND MITOCHONDRIAL DNA

Mitochondria are double membraned organelles where fats and carbohydrates are oxidized and converted to the reducing power of NADH (Nicotinamide adenine dinucleotide) and FADH₂ (Flavin adenine dinucleotide). This reducing power is utilized by series of electrogenic proton pumps called the electron transport chain (ETC) to create an electrochemical proton gradient that drives the ATP synthase pump to synthesize ATP (Adenosine triphosphate). The process by which this proton gradient is created and ATP is synthesized is called the oxidative phosphorylation (OXPHOS) (Maley and Plaut 1954, Jacobs and Sanadi 1955).

Mitochondrial proteins are bi-genomically encoded i.e. they are encoded by both the nuclear and mitochondrial genome (mtDNA) (Beattie, Battie et al. 1980, de Jong, Holtrop et al. 1980, Attardi and Schatz 1988). MtDNA is double-stranded and circular in nature as depicted in **Figure 1** (Nass and Nass 1963). MtDNA is present compacted in the nucleoids and located in the mitochondrial matrix interacting with the inner membrane (Nass and Nass 1963, Nass 1969, Albring, Griffith et al. 1977). With a size of 16,569 base pair (bp), mtDNA encodes for 13 mRNAs, 2 rRNAs and 22 tRNAs, which are required for forming functional Electron transport chain (ETC) in the mitochondria (Anderson, Bankier et al. 1981, Andrews, Kubacka et al. 1999). Depending upon the metabolic capacity and energy demand of the cell, hundreds to thousands of mitochondria can be present (Fernandez-Vizarra, Enriquez et al. 2011), with each mitochondrion having 2-10 copies of the mtDNA (De Giorgi, D'Alessandro et al. 1992, Wiesner, Ruegg et al. 1992). Unlike the nuclear genome, mitochondrial genome is maternally inherited, as mitochondria in sperm get actively eliminated by ubiquitin dependent mechanism (Sutovsky, Moreno et al. 1999, Sutovsky, Moreno et al. 2000).

1.1.2 TRANSCRIPTION OF MTDNA-ENCODED GENES

MtDNA-encoded genes are asymmetrically arranged between the Heavy strand (H-strand) and Light strand (L-strand) and encode for all the complexes of electron transport chain, except Complex II. H strand encodes 12 of the 13 mRNAs, 14 of the 22 tRNAs and 2 rRNAs, whereas the L strand codes for single mRNA - ND6 (subunit of Complex I) and the remaining 8 of the 22 tRNAs as depicted in **Figure 1** (Anderson, Bankier et al. 1981, Attardi and Schatz 1988, Andrews, Kubacka et al. 1999). There are only two non-coding regions (NCR) in the mtDNA that house the regulatory elements for mtDNA replication and transcription. The major non-coding region is 900 bp long and is called the D-loop. D-loop is the region of mtDNA where the promoters for H-strand and L-strand as well as the origin of replication for H-strand (O_H) are located and thus, serves as the major site for transcriptional regulation as represented in **Figure 1 and 2**. The minor NCR is 30 bp long containing O_L (Origin of replication of L strand) and is located between $tRNA^{Cys}$ and $tRNA^{Asn}$, within a cluster of tRNA genes. O_L serves as the site of initiation for lagging-strand synthesis of mtDNA (Shadel and Clayton 1997).

For mitochondrial transcription to be initiated and activated, a complex of proteins such as POLRMT, TFAM and TF2BM form the transcriptional machinery, that bind at the promoters located in the D-Loop (**Figure 2**). POLRMT is the RNA polymerase of mitochondria, whereas TFB2BM and TFAM stand for Transcription factor 2B of mitochondria and Transcription factor A of mitochondria, respectively. There are two promoters for the transcription from the H-strand – HSP1 (H-strand promoter 1) and HSP2 (H-strand promoter 2) that are located within 100 bp of each other and transcribed in the same direction (Montoya, Christianson et al. 1982, Montoya, Gaines et al. 1983). HSP-1 transcript codes for 2 tRNA ($tRNA^{Phe}$ and $tRNA^{Val}$) and 2 rRNAs (12S and 16S rRNA) and gets terminated just downstream of 16S rRNA by the binding of

TERMF1 (Termination factor 1) at tRNA^{Leu} (Kruse, Narasimhan et al. 1989, Roberti, Polosa et al. 2009) (**Figure 2**). But the HSP-2 transcript codes for all the genes present on H-strand (2 rRNAs, 13 tRNAs and 12 mRNAs) and gets terminated at A/T rich area called H2, which is located upstream of tRNA^{Phe} (Camasamudram, Fang et al. 2003). Similar to the HSPs, LSP or L-strand promoter is located within the D-loop but transcription occurs in the opposite direction than that of HSPs. The transcript generated from LSP codes for the genes present on L-strand (ND6 and 8 tRNAs) and gets terminated at a region 160-185 bp downstream of LSP within the D-loop where O_H is located (Bonawitz, Clayton et al. 2006, Scarpulla 2008). Thus, bidirectional transcription of mtDNA results in poly-cistronic transcription units, which are subsequently processed to excise tRNAs, mRNAs and rRNAs. Termination codons are not encoded in mtDNA but are generated by post-transcriptional polyadenylation of corresponding mRNAs (Peralta, Wang et al. 2012). tRNAs are equally interspersed among other genes playing an important role in RNA processing (Ojala, Merkel et al. 1980, Ojala, Montoya et al. 1981). MtDNA-encoded genes are translated using a genetic code that differs from the universal genetic code, as reflected from their initiation and termination codons along with a simplified codon-anticodon pairing system that allows translation to proceed with only 22 tRNAs (Attardi and Schatz 1988).

1.1.3 HOW RETROGRADE SIGNALING CAN DETERMINE MTDNA TRANSCRIPTION

Although mitochondria are bi-genomically encoded where majority of mitochondrial proteins are encoded by the nucleus and only 13 are encoded by mtDNA, mitochondrial function can be governed by regulating the expression of both nuclear-encoded and mitochondrial-encoded mitochondrial proteins. Nuclear-encoded mitochondrial proteins comprise of various subunits of different complexes in the ETC, structural proteins, transcription factors governing

mitochondrial transcription (TFAM, PORMT, TFB2M, TERMF1) and proteins involved in mitochondrial DNA replication (Polymerase γ , Twinkle, mtSSB), all of which are required for mitochondrial maintenance and biogenesis (Scarpulla 2008).

Interestingly, mitochondrial function can also determine the mitochondrial biogenesis via the retrograde signaling pathway, which regulates the transcription of nuclear DNA-encoded proteins that ultimately regulates mitochondrial transcription (Finley and Haigis 2009). The OXPHOS activity of mitochondria determines the levels of ROS (reactive oxygen species), ratios of ATP/ADP and NADH/ NAD⁺ in the cells and mitochondria's ability to store calcium ions determines the cytosolic levels of calcium ions [Ca²⁺]. Each of these parameters- ROS, ATP/ADP, NADH/NAD⁺ and cytosolic [Ca²⁺] - reflect the metabolic status of the cell. ROS can cause redox signaling through p53, ERK, PI(3)K, JNK, p38 MAPK and AKT; ATP levels can trigger the kinase activity of AMPK; NAD⁺ can activate SIRT1's de-acetylase activity whereas the [Ca²⁺] can trigger Calcium signaling and activate CREB by phosphorylating it.

Phosphorylated CREB promotes transcription of PGC1 α , whereas SIRT1-mediated de-acetylation and AMPK-mediated phosphorylation of PGC1 α can stimulate PGC1 α activity.

Activated PGC1 α is a nuclear coactivator of various nuclear transcription factors, such as NRF1, NRF2, ERR- α and PPAR- γ , that are responsible for the transcription of nuclear-encoded mitochondrial proteins including transcription factors required for mitochondrial transcription.

(Finley and Haigis 2009)

1.1.4 MTDNA MUTATIONS, HETEROPLASMY AND IMPLICATIONS OF POSSIBLE ROLE IN AGING AND NEURODEGENERATIVE DISEASES

Importance of mtDNA can be emphasized by citing the example of Rho-zero cells (ρ^0). ρ^0 cells are created by selective depletion of mtDNA. Thus, these cells lack functional ETC and are incapable of oxygen consumption. Normal respiratory function can be restored in the ρ^0 cells by introducing exogenous functional mtDNA. (Miller, Trimmer et al. 1996, Inoue, Takai et al. 1997)

In a given individual, when all mtDNA copies are identical, such a state is referred to as homoplasmy. As mitochondria serve as the prime site for ROS generation, coupled with error-prone Polymerase γ (DNA polymerase of the mitochondria), mutations in mtDNA can arise, be maintained or amplified to different levels. These mutations co-exist with the wild type-mtDNA giving rise to a condition called heteroplasmy. Random distribution of mitochondria occurs during mitosis giving rise to varying degrees of heteroplasmy in different cells or tissues. When this varying degree of heteroplasmy crosses the threshold level, starts manifesting in the form of mitochondrial dysfunction and thus, results in mtDNA disease (Lightowlers, Chinnery et al. 1997, Rossignol, Malgat et al. 1999). Several mtDNA mutations are known to be pathogenic in nature causing multiple disorders such as LHON, MELAS, MERFF, NARP and MILS as depicted in **Table 1**. Multiple studies have reported positive correlations between mtDNA mutations and aging-associated mitochondrial dysfunction observed in various disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), cancer and hypertension (Fujita, Yamauchi et al. 1996, Polyak, Li et al. 1998, Lin, Simon et al. 2002, Simon, Lin et al. 2004). However, a large fraction of mitochondrial diseases cannot be explained by the alteration observed in the genetic sequence of mtDNA (Dimauro and Davidzon 2005).

Understanding how alterations apart from the genetic sequence of mtDNA can affect mitochondrial function is critical and can be of therapeutic value, which remains largely unexplored.

1.2. DNA METHYLATION

1.2.1 FUNCTIONS OF DNA METHYLATION

Addition of methyl group to the C5 position of the cytosine in DNA yields 5-methyl 2'-deoxycytosine (5-mC), which is known as DNA methylation. DNA methylation is one of the epigenetic marks that control gene-expression without any alteration in the DNA sequence itself. Epigenetic marks such as histone modifications and DNA methylation control the chromatin architecture by regulating the access of DNA-binding proteins to DNA, which are involved in chromatin remodeling. Chromatin comprises of the genomic DNA associated with the nucleosome core histone proteins (H2A, H2B, H3 and H4) and the linker histone (H1) where nucleosome is the fundamental subunit of chromatin. Nucleosome is made up of DNA coiled twice around a histone octamer. The core histone proteins have modifications such as acetylation at lysine residues and methylation at arginine or lysine residues. Histone acetyl transferase (HAT) and histone deacetylase (HDAC) are responsible for regulating histone acetylation whereas Histone methyl transferase (HMT) and Histone demethylase (HDM) regulate histone methylation. Repressor complexes such as HDAC and HMT are recruited by methyl-DNA-binding proteins (MBP) that are known to bind methylated CpG sites in the DNA. This recruitment of HDAC and HMT by MBP bound at a methylated CpG site, leads to creation of repressive marks on histone (de-acetylation and methylation) and establishes a chromatin architecture where gene expression is repressed. (Ndlovu, Denis et al. 2011, Jones 2012)

Most work in animals regarding DNA methylation have been focused on 5-methyl cytosine (5-mC) occurring in CpG islands (CGI) at transcriptional start sites (TSS), but it's the position of the 5-mC in the transcriptional unit that exerts its effects on gene expression (Jones 2012). If 5-mC occurs in TSS then it blocks the initiation of transcription (Kass, Landsberger et al. 1997, Hashimshony, Zhang et al. 2003, Kelly, Miranda et al. 2010), whereas if it occurs within the gene body (Wolf, Jolly et al. 1984), it might stimulate transcription elongation (Hellman and Chess 2007, Feng, Cokus et al. 2010) or might impact splicing (Schwartz, Meshorer et al. 2009, Shukla, Kavak et al. 2011). 5-mC is also observed in repeat regions or transposable elements where it is known to suppress their transcription and thus, play a role in genome stability (Yoder, Walsh et al. 1997).

Non-CpG methylation can be defined as methylation of cytosines that occur outside of CpG sites, within CpC, CpT and CpA or CCWGG (W=A/T) or CpNpG (N=A/T/C/G). Non-CpG methylation is found in plants (Vanyushin and Ashapkin 2011), bacteria (Militello, Simon et al. 2012), fungi (Goyon, Barry et al. 1996, Goyon, Rossignol et al. 1996) and has been recently reported in mammals (Tomizawa, Kobayashi et al. 2011, Ziller, Muller et al. 2011, Ichiyanagi, Ichiyanagi et al. 2013, Varley, Gertz et al. 2013). Non-CpG methylation in mammals was first seen in murine embryonic stem cells (ESC) (Ramsahoye, Biniszkiwicz et al. 2000). Although levels of non-CpG methylation are low in somatic tissues, it has been extensively found in postnatal human and mouse brain (Varley, Gertz et al. 2013). In ESC, non-CpG methylation has been found to correlate with gene expression (Ramsahoye, Biniszkiwicz et al. 2000). However, an inverse correlation was seen with transcription in neurons (Varley, Gertz et al. 2013). Thus, the function of mammalian non-CpG methylation is not clear and appears to be context dependent in nature.

1.2.2 DNA METHYL TRANSFERASES

DNA methyl transferases (DNMTs) transfer the methyl group from a cofactor molecule S-Adenosyl-L-methionine (SAM) to the C5 position of the cytosine in DNA yielding 5-methyl 2'-deoxy-cytosine (5-mC) and S-Adenosyl-L-homocysteine (SAH). The four members of mammalian DNMT family are DNMT1, DNMT3A, DNMT3B and the catalytically inactive regulatory factor DNMT3L. All the DNMTs are comprised of a large N terminal domain of variable size (containing regulatory domains involved in interaction with DNA, chromatin or other proteins) and a C-terminal catalytic domain, which is responsible for the methyl transferase activity (Bestor 2000, Jurkowska, Jurkowski et al. 2011). Unlike DNMT1 that has a strong preference for hemi-methylated sites in DNA (Goyal, Reinhardt et al. 2006), DNMT3A and DNMT3B does not have a preference between hemi-methylated and un-methylated DNA (Okano, Xie et al. 1998). Methylation patterns are established in the developing embryo by *de novo* DNA methyl transferases- DNMT3A and DNMT3B (Okano, Bell et al. 1999). For maintenance, these methylation marks are predominantly copied onto the nascent strand by DNMT1, along with cooperation of DNMT3A and DNMT3B (Jones and Liang 2009). Physiological importance of DNMTs can be emphasized by the fact that deletion of either of *Dnmt1* or *Dnmt3b* is embryonically lethal, while *Dnmt3a* null mice survive for 3-4 weeks only after birth (Li, Bestor et al. 1992, Okano, Bell et al. 1999). Mice knocked out for any of these above-mentioned *Dnmts* have severe developmental defects.

Until very recently, DNMTs were known to be involved in methylation of only CpG sites in the mammalian genome. Recent reports suggest that DNMTs can also methylate non-CpG sites. DNMT3A and DNMT3B are responsible for CpC, CpT and CpA methylation exclusively in

embryonic stem cells (ESC) (Ramsahoye, Biniszkiewicz et al. 2000, Ziller, Muller et al. 2011, Arand, Spieler et al. 2012), whereas DNMT1 was found to methylate 5' Cytosine in CpC, CpT and CpA in haploid sperm and somatic tissues (Grandjean, Yaman et al. 2007).

1.2.3 DISEASE RELEVANCE OF DNA METHYLATION

Abnormalities in DNA methylation has been found in a wide range of diseases. DNA methylation is essential for genomic imprinting where paternal or maternal allele gets silenced by the dint of methylation. Defects in genomic imprinting are involved in Prader-Wili syndrome, Angleman syndrome, Beckmith-Widerman syndrome and some cancers (Paulsen and Ferguson-Smith 2001). DNA methylation profiles of genes involved in Alzheimer's disease (AD) such as Apo lipoprotein E (APOE) and presenelin 1 (PSEN1) differ distinctly in bio-specimens between that of AD patients and age-matched control subjects (Wang, Oelze et al. 2008). Similarly, decreased levels of methylation were found in α -synuclein (SNCA) gene in bio-specimens derived from patients with idiopathic Parkinson's disease (Jowaed, Schmitt et al. 2010, Matsumoto, Takuma et al. 2010). Even during aging, global hypo-methylation and gene-specific hyper-methylation are observed and are increasingly reported to be associated with age-related diseases (Vanyushin, Nemirovsky et al. 1973, Romanov and Vanyushin 1981, Gonzalo 2010, D'Aquila, Rose et al. 2013). Hypo-methylation due to mutations in the DNMT3B causes the autosomal dominant recessive Immunodeficiency Centromere instability and Facial anomalies (ICF) syndrome (Hansen, Wijmenga et al. 1999, Ehrlich, Buchanan et al. 2001). Thus, the correct establishment and maintenance of DNA methylation patterns are critical for mammalian development and normal cellular function.

1.3. MITOCHONDRIAL DNA METHYLATION

1.3.1 EARLY STUDIES OF MTDNA METHYLATION

MtDNA methylation and DNA methylase activity in mitochondria was first reported in loach embryos using Thin Layer Chromatography (TLC)-radiography in 1971 (Vanyushin, Kiryanov et al. 1971). By using similar approaches, a subsequent study found mtDNA to have low but a significant percentage of methylation of 0.2-0.6% in cultured mouse fibroblasts and baby hamster kidney cells (Nass 1973). Another study used spectrophotometric determination after resolving hydrolyzed DNA by TLC to determine the levels of 5-mC and reported that mtDNA from livers of sheath-fish r, duck, chicken, rat, ox and ox heart have mtDNA methylation at mole percent of 3.03, 1.52, 1.63, 2.0, 3.03 and 3.15, respectively (Vanyushin and Kirnos 1977). However, a single study reported mtDNA methylation levels to be below their detection limit in frog ovary and HeLa cells using TLC, thus claiming mtDNA methylation to be absent (Dawid 1974).

Later, Waalwijk and Flavell adapted usage of restriction endonucleases– MspI and HpaII – to determine the presence of methylation at CCGG, which is now commonly called the methylation-sensitive restriction enzyme (MSRE) analysis (Waalwijk and Flavell 1978). Coupling MSRE analysis with Southern blot and radiography, two independent studies reported similar percentage (2-5%) of CCGG sites of mtDNA to be methylated in HeLa, human skin fibroblast and Ltk⁻ APRT⁻ mouse cells (Shmookler Reis and Goldstein 1983, Pollack, Kasir et al. 1984). However, a single study claimed mtDNA methylation to be absent when they did not observe a difference in digestion pattern using semi-quantitative ethidium bromide staining after MSRE-hydrolyzed mtDNA was resolved in an agarose gel (Groot and Kroon 1979).

Thus, using both the approaches – TLC and MSRE analysis, seven independent studies were carried out to test the presence and levels of mtDNA methylation. For both the approaches, five studies reported mtDNA methylation to be present (albeit at varying amounts) whereas two asserted its absence. Due to all these inconsistent reports about the level of mtDNA methylation and also two studies claiming its absence, mtDNA methylation remained controversial since its inception.

1.3.2 RESURGENCE IN THE FIELD OF MTDNA METHYLATION

Even though significant advances were made in understanding how DNA methylation and histone-modifications control nuclear gene expression, mtDNA methylation remained ignored for almost four decades, primarily because of dearth of sensitive methods for studying mtDNA methylation. Although, DNA methylase activity was known to occur in mitochondrial fractions (Vanyushin, Kiryanov et al. 1971, Nass 1973, Vanyushin and Kirnos 1977), but no DNA methyl transferases were known to be present in mitochondria. Neither were “histones-like” proteins or structures known in mitochondria.

Localization of DNMTs in mitochondria:

Recently, existence of mitochondrial isoform of DNMT1 (mtDNMT1) was demonstrated by Shock *et. al.* in HCT116 human carcinoma cell line and mouse embryonic fibroblast (MEF) (Shock, Thakkar et al. 2011), revitalizing interest in studying mtDNA methylation. MtDNMT1 was found to be transcribed from two possible start sites located upstream of the canonical one. Moreover, mtDNMT1 was found to translocate into the mitochondrial matrix and bind mtDNA. MtDNMT1 expression was found to be sensitive to the transcription factors that control

mitochondrial biogenesis- PGC1- α and NRF1 - during oxidative stress. After this initial study (Shock, Thakkar et al. 2011), several *in vivo* and *in vitro* studies have independently reported the presence of DNMTs in mitochondria as discussed below.

DNMT1 in mitochondria: DNMT1 localization in mitochondria has been found in both human cell lines (HeLa (Bellizzi, D'Aquila et al. 2013) and HCT116 colon carcinoma (Shock, Thakkar et al. 2011)) and bio-specimens derived from motor cortex of ALS patients (Chestnut, Chang et al. 2011). In murine model, mitochondrial localization of DNMT1 was detected *in vitro* (NSC34 mouse motor neuron cell line, MEF (Shock, Thakkar et al. 2011) and 3T3-L1 fibroblast-like cells (Bellizzi, D'Aquila et al. 2013)), but not *in vivo*, in the adult organs such as brain, spinal cord (Chestnut, Chang et al. 2011) and skeletal muscle (Wong, Gertz et al. 2013).

DNMT3A in mitochondria: DNMT3A localization in mitochondria is reported in post-mortem bio-specimens of cerebral cortex and specifically the motor cortex of ALS patients (Chestnut, Chang et al. 2011). However, DNMT3A was not detected in mitochondria of cell lines - HeLa (Bellizzi, D'Aquila et al. 2013) and HCT116 colon carcinoma (Shock, Thakkar et al. 2011). In the mouse mitochondria, DNMT3A has been detected in NSC-34 motor neuron cell line (Chestnut, Chang et al. 2011) as well as in several adult tissues (brain, synapses and spinal cord (Chestnut, Chang et al. 2011, Wong, Gertz et al. 2013), heart, testes, skeletal muscle (Wong, Gertz et al. 2013)). However, mitochondrial DNMT3A was not found in 3T3-L1 fibroblast-like cells (Bellizzi, D'Aquila et al. 2013), mouse embryonic fibroblasts (MEF) (Shock, Thakkar et al. 2011) and various organs in mouse (spleen, liver, lung) (Wong, Gertz et al. 2013).

DNMT3B in mitochondria: So far, mitochondrial localization of DNMT3B has been investigated only *in vitro* conditions. DNMT3B was detected in mitochondria of HeLa and 3T3-L1 cells

(Bellizzi, D'Aquila et al. 2013), but not in mitochondria of HCT116 colon carcinoma and MEF (Shock, Thakkar et al. 2011).

Thus, the expression of DNMTs in mitochondria is emerging to be cell-line specific as well as tissue and species-specific in nature. Even though all these studies have reported presence of DNMTs in mitochondria, whether DNMTs are involved in mtDNA methylation is not known.

Emerging histone-like role for TFAM:

TFAM belongs to the family of High Mobility Group (HMG) proteins and contains two HMG-Box domains which are known to bind DNA and are characteristic of proteins that regulate DNA-dependent processes such as transcription and replication (Zhang and Wang 2010). TFAM binds mtDNA in both sequence-specific and non-specific manner. TFAM binds at HSP-1 and LSP in a sequence-specific manner and is responsible for bending DNA, allowing interaction with transcription machinery, and thus, initiate transcription (Ngo, Kaiser et al. 2011). Also, non-specific binding of TFAM in mtDNA, is reported to be responsible for looping and finally compaction of mtDNA into nucleoids. Thus, TFAM determines the “ON” state of mtDNA during transcription from HSP-1 and LSP; and also can lead to compacted mtDNA as observed during “OFF” state in the nucleus. (Ngo, Lovely et al. 2014)

Post-translational modifications of TFAM can alter its binding affinity for mtDNA and thus, regulate transcription. One group reported that ERK1/2-mediated phosphorylation of TFAM decreased the binding affinity of TFAM at LSP and thus mitochondrial transcription (Wang, Zhu et al. 2014). Another study reported that ubiquitination of TFAM impedes its transport to the mitochondria resulting in subnormal mtDNA transcription (Santos, Mishra et al. 2014). In line

with above studies, phosphorylation of TFAM in HMG box 1 (HMG1) by cAMP-dependent protein kinase in mitochondria impaired TFAM's ability to bind mtDNA and to activate transcription (Lu, Lee et al. 2013). Thus, similar to histones, TFAM can get post-translationally modified and affects mtDNA transcription.

In short, a histone-like role of TFAM has been emerging which raises speculations about the possibility of epigenetic control of mtDNA.

1.3.3 POSSIBLE FUNCTIONAL RELEVANCE OF MTDNA METHYLATION

Since the discovery of DNMTs in mitochondria (Chestnut, Chang et al. 2011, Bellizzi, D'Aquila et al. 2013, Wong, Gertz et al. 2013) (Shock, Thakkar et al. 2011), several studies have reported alterations of mtDNA methylation at D-loop as well as within genes in multiple pathological and physiological conditions as explained below.

Variations in mtDNA methylation in pathological conditions:

Alteration in mtDNA methylation has been correlated with a multitude of diseases. Recently, a study reported decrease in global mtDNA methylation in the mtDNA isolated from EBV-immortalized lymphoblastoid cells of children with Down's syndrome in comparison to that of healthy age-matched subjects (Infantino, Castegna et al. 2011). Another study conducted using bio-specimens of human motor cortex found that localization of both DNMT1 and DNMT3A in mitochondria were increased along with an increase in mitochondrial staining of 5-methyl cytosine in ALS patients (Chestnut, Chang et al. 2011). Another group reported elevated levels of MT-ND2 in colon and rectal cancer bio-specimens, which was correlated with decreased D-loop methylation in comparison to their corresponding non-cancerous tissues (Feng, Xiong et al.

2012). Additionally, MT-ND6 methylation was found to be elevated in mtDNA from liver of patients having non-alcoholic steatohepatitis (NASH) in comparison to simple steatosis, which was accompanied by significantly reduced transcription of MT-ND6 and increased DNMT1 mRNA levels in liver of NASH subjects (Pirola, Gianotti et al. 2013). Further, platelet mtDNA methylation was significantly higher in cardiovascular disease patients than healthy control subjects at MT-CO1, MT-CO2, MT-CO3 and MT-TL1 (Baccarelli and Byun 2015). Moreover, D-loop methylation has also been reported to be significantly increased in the mtDNA derived from buffy coat of obese subjects in comparison to lean subjects and in insulin-resistant subjects when compared to insulin-sensitive subjects (Zheng, Linarelli et al. 2015). In a single study conducted in mouse model for pre-symptomatic ALS, mitochondrial DNMT3A were significantly reduced in skeletal muscle and spinal cord, which was accompanied by increased in MT-RNR2 methylation in the spinal cord (Wong, Gertz et al. 2013).

Variations in mtDNA methylation with physiological and environmental conditions:

Several studies have also investigated if mtDNA methylation levels change during various developmental stages. Significant reduction of mtDNA methylation was observed in MT-ND6 and MT-ATP6 with progressing developmental stages in human brain (Ghosh, Sengupta et al. 2014). Further, mtDNA methylation at D-loop and MT-RNR1 was significantly different between the bio-specimens of cord blood and placental tissue (Janssen, Byun et al. 2014). Similar developmental-stage specific changes in mtDNA methylation have been reported in mouse studies. For instance, significantly lower levels of mtDNA methylation were observed in mouse somatic tissues in comparison to murine ESC at various regions in the mtDNA- D-loop, tRNA/COXI and CytB (Kelly, Mahmud et al. 2012).

Few studies have also correlated mtDNA methylation with exposure levels of airborne-environmental toxicants. MtDNA methylation was studied in buffy coat of human subjects with low and high exposure of metal-rich particulate matter. MT-TF and MT-RNR1 regions in mtDNA were found to have significantly higher methylation in subjects with higher metal exposure (Byun, Panni et al. 2013). In a separate study, placental mtDNA methylation at MT-RNR1 and D-loop was found to be positively-associated with higher levels of exposure to metal-rich particulate matter (Janssen, Byun et al. 2015).

In summary, several recent studies reported changes in mtDNA methylation in bio-specimens obtained from several physiological states and pathophysiological conditions. A few of these studies have also reported concomitant changes in expression levels of some of the mtDNA-encoded genes. However, any causal significance of these changes in mtDNA methylation with regard to mitochondrial gene expression and function remains to be explored.

1.3.4 IS MITOCHONDRIAL DNA METHYLATION STILL DUBIOUS?

Several recent studies have claimed the presence of mtDNA methylation in different model systems and reported changes in mtDNA methylation in multiple pathological and physiological conditions as described above. However, few other studies asserted mtDNA methylation to be absent or present at non-significant levels (Dawid 1974, Groot and Kroon 1979, Maekawa, Taniguchi et al. 2004, Hong, Okitsu et al. 2013). Thus, the debate revolving presence of mtDNA methylation still has not ended completely. All the methods that have been used to study mtDNA methylation have some technical limitations that have led to reproducibility issues and risk of generating artifacts. Further, majority of the studies have not considered the possibilities of nuclear DNA contamination for studying mtDNA methylation and thus not used mtDNA

extracted from purified mitochondria for determining the presence of mtDNA methylation or characterized mtDNA purity extensively. Thus, a major limitation common for all the methods that have been used to study mtDNA methylation is the possibility of nuclear DNA contamination, which need to be addressed in order to accurately predict the levels of mtDNA methylation (van der Wijst and Rots 2015).

In 1970's, mtDNA methylation studies was studied primarily by growing cells in medium containing radiolabeled substrates solely or in combination such as ^{14}C , ^3H or ^{32}P , followed by hydrolysis of DNA and subsequent resolution by thin-layer chromatography and radiographic or spectrophotometric detection (Vanyushin, Kiryanov et al. 1971, Nass 1973, Dawid 1974, Vanyushin and Kirnos 1974, Vanyushin and Kirnos 1977). Incomplete DNA hydrolysis, inability to resolve relatively small fraction of modified cytosines from the parent cytosine, detection limit being higher than the actual amount present in test-sample and possibility of losing the radioactive signal were the limitations of this approach, which led to reproducibility issues.

In 1978, Waalwijk and Flavell demonstrated the use of restriction endonucleases – MspI and HpaII – by exploiting the differential preference of cleaving at the internal cytosine in the recognition sequence – CCGG, to determine the presence of methylation (Groot and Kroon 1979, Shmookler Reis and Goldstein 1983, Pollack, Kasir et al. 1984). Digestion products of the mtDNA by MspI and HpaII were run on an agarose gel, transferred on a nitrocellulose membrane and radiolabeled oligonucleotide probes were used to detect the presence of fragment of desired size depending upon the presence or absence of 5-methyl cytosine at CCGG. Using this approach, both Shmookler et. al 1983 and Pollack et. al 1984 have reported 2-5% methylation at CCGG sites in mtDNA from HeLa, primary human skin fibroblasts and LTK⁻

Aprt⁻ mouse cells. On the other hand, in another study when the digested products of rat and calf liver mtDNA were just run on an agarose gel, no differences were found, possibly due to the “semi-quantitative” nature of agarose gel. The major limitations of this approach are that study of mtDNA methylation is restricted to a particular site CCGG only and also that the presence of incompletely digested or indigestible mtDNA cannot be ruled out.

With advances in technology for studying DNA methylation since 1980's, mtDNA methylation has been pursued by multiple approaches such as methylation-specific PCR and bisulfite-sequencing post bisulfite-conversion and various antibody-based methods such as immunohistochemistry, methylated DNA-IP (Me DIP) and ELISA (Enzyme-linked Immunosorbent assay)).

Antibody-based methods require binding of antibody at target sites, but as it is difficult to determine the efficiency of binding of antibodies to their target sites, percentage pulldown of the input DNA cannot be predicted accurately using Me DIP. Further, in 5-mC-ELISA, the frequency of methylation is quantitated using a control standard of unknown proprietary sequence available in the 5-mC-ELISA commercial kits and hence, does not provide the real frequency of methylation. Additionally, chances of non-specific binding of the antibody cannot be eliminated completely, which holds true for all the antibody-based methods that have been used to study mtDNA methylation. (Shock, Thakkar et al. 2011, Dzitoyeva, Chen et al. 2012, Kelly, Mahmud et al. 2012, Bellizzi, D'Aquila et al. 2013, Wong, Gertz et al. 2013)

Multiple technical limitations are present even for bisulfite-conversion based methods. The process of bisulfite-conversion is harsh and causes DNA degradation. Also, the efficiency of bisulfite-conversion needs to be addressed for accurate quantification. Moreover, multiple

studies have reported the presence of 5-hydroxymethyl cytosine (5-hmC) in mtDNA (Shock, Thakkar et al. 2011, Dzitoyeva, Chen et al. 2012, Bellizzi, D'Aquila et al. 2013). Thus, detection of 5-mC in mtDNA is probably confounded with the presence of 5-hmC as bisulfite-conversion cannot distinguish between modified cytosines such as 5-methyl cytosine and 5-hydroxymethyl cytosine. Additionally, low-coverage issues during next-generation sequencing and Polymerase Chain Reaction (PCR)-jackpot effect that might occur during PCR amplification before sequencing are additional limitations that need to be carefully considered for accurate quantification of mtDNA methylation. (Maekawa, Taniguchi et al. 2004, Feng, Xiong et al. 2012, Byun, Panni et al. 2013, Hong, Okitsu et al. 2013, Pirola, Gianotti et al. 2013, Ghosh, Sengupta et al. 2014, Janssen, Byun et al. 2014, Baccarelli and Byun 2015, Janssen, Byun et al. 2015)

A single study has investigated mtDNA methylation using high pressure liquid chromatography in tandem with mass spectrophotometric quantification (HPLC-MS/MS) after digesting DNA into nucleosides and detected 5mC in mtDNA (Infantino, Castegna et al. 2011). Although HPLC-MS/MS does not provide the information for site specific methylation, it is the most-direct, specific and very sensitive method to detect global methylation (Dahl and Guldberg 2003). Unlike methylation-specific PCR and bisulfite sequencing that require bisulfite conversion of the DNA or various antibody-based methods that require binding of the antibody, the digested DNA can be directly used for quantification of 5-mC. Unlike methods dependent on bisulfite-conversion, HPLC-MS/MS can distinguish between modified cytosines (5-mC or 5-hmC) in the mtDNA, thus making HPLC-MS/MS the most specific method. However, incomplete DNA digestion, inter-sample variation in digestion efficiency, multiple-steps involved in digesting DNA and the likelihood of nuclear DNA contamination can introduce

variabilities and thus lead to inaccurate prediction of 5-mC in mtDNA (van der Wijst and Rots 2015). Thus, these limitations need to be addressed for accurate quantification of global levels of mtDNA methylation using HPLC-MS/MS.

1.4. AGING-ASSOCIATED MITOCHONDRIAL DYSFUNCTION - ROLE OF MTDNA, DNA METHYLATION AND MTDNA METHYLATION IN AGING

1.4.1 MTDNA IS ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION DURING AGING

Aging-associated mitochondrial dysfunction such as decline in respiration and appearance of abnormal mitochondria and alterations in mtDNA such as deletions, point-mutations and tandem duplications have been observed in various organs in animal models (Vermulst, Wanagat et al. 2008) and clinical samples (Short, Bigelow et al. 2005). During aging, mtDNA mutations are known to accumulate (Trifunovic 2006), which are attributed to oxidative damage caused by elevated levels of reactive oxygen species (ROS) (Harman 1956, Harman 1981), the poor proof-reading efficiency of polymerase- γ (Kaguni 2004, Trifunovic, Wredenberg et al. 2004, Pursell, McDonald et al. 2008, Vermulst, Wanagat et al. 2008) and also to the limited efficiency of the mtDNA repair machinery (Druzhyna, Wilson et al. 2008, Santos, Correia et al. 2013). Further, mitochondrial dysfunction that occurs during aging is also well established. However, as mitochondrial function is regulated by both the nuclear and mitochondrial genome, whether alterations in nuclear genome or mtDNA are responsible for age-associated mitochondrial dysfunction, cannot be concluded. Although, several studies have reported strong correlation of mtDNA mutations with age-associated decline in oxygen consumption and tissue-dependent decrease in enzymatic activities of ETC, there is lack of

evidence that support a direct role of mtDNA in aging-associated mitochondrial dysfunction (Bratic and Larsson 2013).

1.4.2 CYTOPLASMIC HYBRID SYSTEM AND STUDIES ON DIRECT ROLE OF MTDNA IN MITOCHONDRIAL DYSFUNCTION

Cytoplasmic hybrid (cybrid) cell lines are created by transferring subject's mitochondria in an mtDNA-depleted cell line and thus, perpetuate mitochondrial DNA (mtDNA)-encoded components of the subject. Since the nuclear background of different cybrid lines is kept constant with only difference being the source of mtDNA, this technique allows investigators to study the influence of mtDNA on various cellular functions. Prior usage of cybrids has elucidated the contribution of mtDNA to various parameters associated with mitochondrial function such as electron transport chain activities, bioenergetic fluxes, free radical production and mitophagy in various disease models (Swerdlow, Parks et al. 1997, Esteves, Domingues et al. 2008, Esteves, Lu et al. 2010, Swerdlow 2012, Silva, Selfridge et al. 2013).

Very few studies have interrogated the direct role of human mtDNA in aging-associated mitochondrial dysfunction using cytoplasmic or nuclear hybrid model and have arrived at conclusions contradictory to each other (Hayashi, Ohta et al. 1994, Laderman, Penny et al. 1996, Isobe, Ito et al. 1998). All these studies used human mtDNA from skin fibroblasts, but used different mtDNA-depleted cell lines and different model systems. While one group concluded that damage in the mtDNA was responsible for the observed differences in aging-dependent functional alterations of mitochondria (Laderman, Penny et al. 1996), the other group inferred that the nuclear genome is responsible for age-related respiration deficiency (Hayashi, Ohta et al. 1994, Isobe, Ito et al. 1998). The cybrid study that reported the role of mtDNA, was

conducted using clones obtained after generating the transformed cybrid cell line from individuals of varied age and found only 5% of the clones obtained from the old individuals were respiration-deficient (Laderman, Penny et al. 1996). The studies which showed role of nuclear genome worked with clones of a very small group of individuals (Hayashi, Ohta et al. 1994, Isobe, Ito et al. 1998). Further, very limited parameters pertinent to mitochondrial dysfunction were investigated in all these studies. Hence, a direct role of human mtDNA in age-associated mitochondrial dysfunction has not been established yet and further comprehensive studies are required in this regard.

1.4.3 DNA METHYLATION AND MTDNA METHYLATION IN AGING

Global hypo-methylation and local hyper-methylation is known to occur upon aging in the nuclear genome (Gonzalo 2010, D'Aquila, Rose et al. 2013). The first study that reported a global loss of DNA methylation during aging was conducted using rat brain and heart (Vanyushin, Nemirovsky et al. 1973). Loss of DNA methylation was subsequently corroborated by multiple studies in different tissues (Romanov and Vanyushin 1981) from rat, mouse and cow, in primary fibroblasts (Wilson and Jones 1983) derived from mice, hamsters and human and bio-specimens such as human lymphocytes (Drinkwater, Blake et al. 1989) and peripheral blood cells (Fuke, Shimabukuro et al. 2004). Also, an age-associated decrease of DNA methylation in inactive X chromosome was seen in peripheral blood cells (Busque, Mio et al. 1996). Such hypo-methylation predominantly affects non-island CpGs (Christensen, Houseman et al. 2009) and interspersed repetitive sequences (IRSs) (Jintaridth and Mutirangura 2010) during aging.

Paradoxically, significant hyper-methylation is observed at the promoters of specific genes upon aging (Christensen, Houseman et al. 2009, Winnefeld and Lyko 2012, Gentilini, Mari et al. 2013). Such age-associated hyper-methylation has been seen in the promoters for tumor suppressor genes such as COX7A1, LOX, RUNX3, RASSF1 and DUSP22. Multiple genes involved in development and growth (IGF2, c-FOS), cell adhesion (CDH1), metabolism (SLC38A4, SLC22A18, ECRG4, ATP13A4, LEP) and DNA repair (MLH1) also reported to have age-associated increase in DNA methylation (Gonzalo 2010, D'Aquila, Rose et al. 2013).

Recently few studies have found total DNA methylation to be different in cybrids created from different haplogroups of human mtDNA, thus implying that mtDNA-specific interactions between mitochondria and the nucleus can regulate epigenetic marks such as DNA methylation. Cybrids created from J haplogroup donors were found to have higher global methylation and lower ATP and ROS levels in comparison to cybrids generated from non-J haplogroup donor (Bellizzi, D'Aquila et al. 2012). Another study also reported differences in global DNA methylation when cybrids created from J and H haplogroup donors were compared (Atilano, Malik et al. 2015). However, whether aging-associated alterations in mtDNA can affect the levels of nuclear DNA methylation is not known.

As discussed above, nuclear DNA methylation has been extensively studied during aging. However, very limited data is available about age-dependent changes in the mtDNA methylation. Dzitoyeva et. al. compared global levels of mtDNA methylation between young and old mice using 5-mC-ELISA in different parts of the brain (frontal cortex and cerebellum) and found them to be comparable (Dzitoyeva, Chen et al. 2012). However, another study analyzed mtDNA methylation using real-time PCR on bisulfite-treated DNA from human blood sample and found

a correlation between 12S rRNA methylation and aging (Iacobazzi, Castegna et al. 2013). Thus, further studies are required to investigate alteration in mtDNA methylation during aging.

1.5. KNOWLEDGE GAPS, SIGNIFICANCE AND PURPOSE OF THE STUDY

How nuclear DNA methylation determines gene-expression of nuclear DNA-encoded genes is fairly known (Jones 2012). However, existence of mtDNA methylation is still debated (van der Wijst and Rots 2015). Moreover, the role of mtDNA methylation in regulating expression of mtDNA-encoded genes and ultimately mitochondrial function remains unexplored. Studying mtDNA methylation can prove to be useful in understanding mitochondrial diseases as alteration in the genetic sequence of mtDNA accounts for only a small fraction of these diseases (Dimauro and Davidzon 2005). The purpose of this dissertation was to establish a method to quantitate mtDNA methylation globally and evaluate the presence of mtDNA methylation in SH-SY5Y neuroblastoma cells; further, to examine whether mtDNA methylation can be altered experimentally and also if there are any associated changes in mitochondrial transcription and function; and finally, to investigate direct role of mtDNA in mitochondrial dysfunction during aging using cybrid model and if mtDNA methylation is also altered in this model.

Although numerous studies investigating mtDNA methylation claimed its presence (Vanyushin, Kiryanov et al. 1971, Nass 1973, Vanyushin and Kirnos 1977, Shmookler Reis and Goldstein 1983, Pollack, Kasir et al. 1984, Chestnut, Chang et al. 2011, Infantino, Castegna et al. 2011, Shock, Thakkar et al. 2011, Dzitoyeva, Chen et al. 2012, Feng, Xiong et al. 2012, Kelly, Mahmud et al. 2012, Bellizzi, D'Aquila et al. 2013, Byun, Panni et al. 2013, Jia, Li et al. 2013, Pirola, Gianotti et al. 2013, Wong, Gertz et al. 2013, Ghosh, Sengupta et al. 2014, Baccarelli and Byun 2015, Janssen, Byun et al. 2015, Mishra and Kowluru 2015, Zheng, Linarelli et al. 2015), a

few have asserted it to be absent or present at non-significant levels (Dawid 1974, Groot and Kroon 1979, Maekawa, Taniguchi et al. 2004, Hong, Okitsu et al. 2013). Methods used to study mtDNA methylation have multiple technical limitations which have led to issues of reproducibility (van der Wijst and Rots 2015). Moreover, majority of these studies have not addressed the possibility of nuclear DNA contamination while assessing the presence of mtDNA methylation and neither did majority of the studies use purified mitochondria (van der Wijst and Rots 2015). Hence, critical evaluation for presence of mtDNA methylation, by addressing the limitations of the method used and issues of nuclear DNA contamination, is required. Here, we aim to establish a direct and specific HPLC-MS/MS based method to quantify global mtDNA methylation and to investigate the occurrence of mtDNA methylation in SH-SY5Y neuroblastoma along with purity characterization of mtDNA.

Recent studies have demonstrated localization of DNMTs in mitochondria (Chestnut, Chang et al. 2011, Shock, Thakkar et al. 2011, Bellizzi, D'Aquila et al. 2013, Wong, Gertz et al. 2013). However, direct role of DNMTs in altering mtDNA methylation is yet to be established. Further, several studies have reported changes in levels of mtDNA methylation in various pathological and physiological conditions. Few of these studies have also reported correlative changes in expression levels of mtDNA-encoded genes (Feng, Xiong et al. 2012, Pirola, Gianotti et al. 2013). However, any role of mtDNA methylation in controlling gene expression of mtDNA-encoded genes and mitochondrial function is not yet tested. In order to test this, experimental approaches to alter mtDNA methylation need to be developed first, as it is not yet known whether mtDNA methylation can be altered experimentally. Hence, we investigated if mtDNA methylation can be altered by different experimental approaches and whether there are any associated changes in mtDNA transcription and mitochondrial function.

Global hypo-methylation (Vanyushin, Nemirovsky et al. 1973, Romanov and Vanyushin 1981, Wilson and Jones 1983, Drinkwater, Blake et al. 1989, Busque, Mio et al. 1996, Fuke, Shimabukuro et al. 2004, Christensen, Houseman et al. 2009, Jintaridth and Mutirangura 2010) and gene-specific hyper-methylation (Christensen, Houseman et al. 2009, Winnefeld and Lyko 2012, Gentilini, Mari et al. 2013) are known to occur during aging. However, whether mtDNA methylation gets altered upon aging is not well known. Although, positive correlations between mtDNA mutations and aging-associated decline in mitochondrial dysfunction exist (Bratic and Larsson 2013), direct role of mtDNA in aging-associated mitochondrial dysfunction is also unclear (Hayashi, Ohta et al. 1994, Laderman, Penny et al. 1996, Isobe, Ito et al. 1998). Hence, we studied whether a direct role of mtDNA exists in aging-associated mitochondrial dysfunction using cybrid model of aging and also how global methylation and mtDNA methylation are affected in this model.

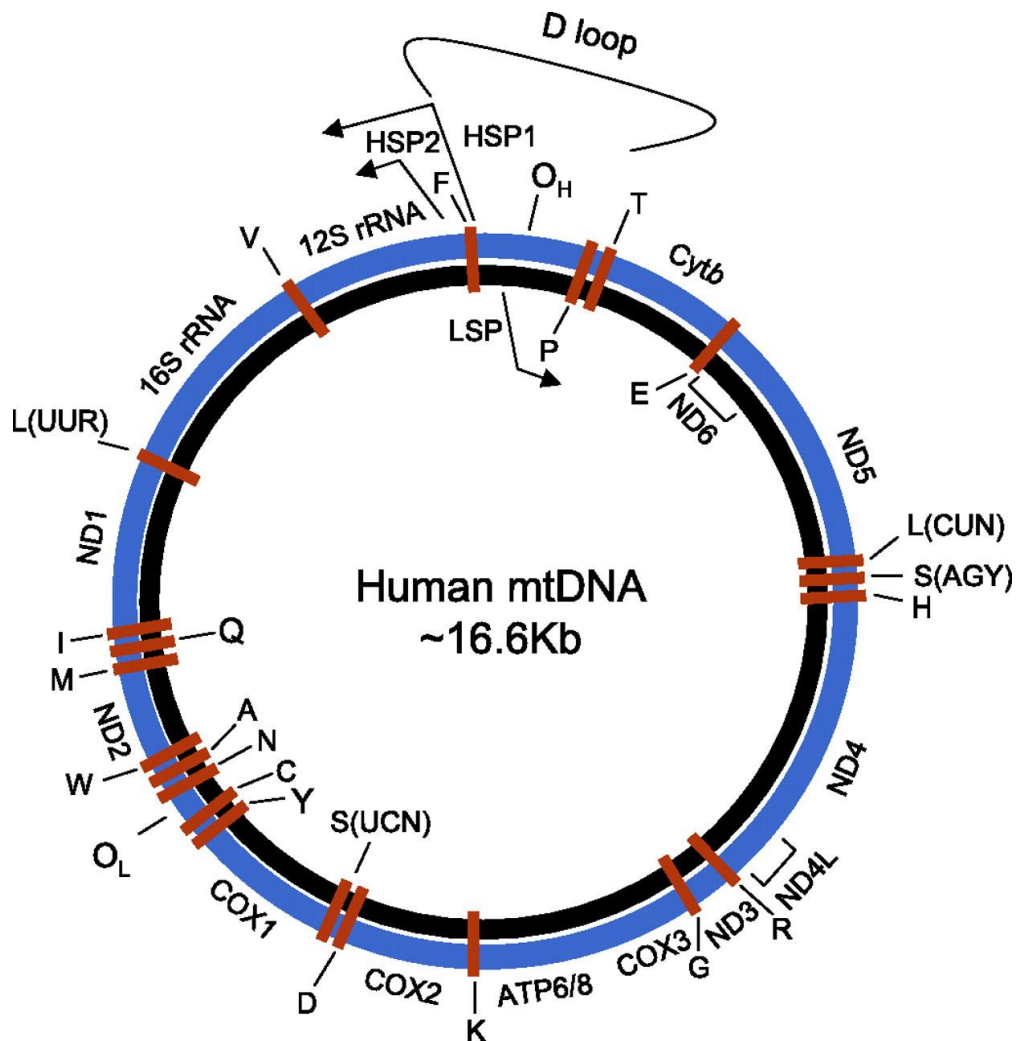


Figure 1: Human mitochondrial DNA and its structural arrangement: MtDNA is double-stranded and circular with as size of 16,569 bp. Blue strand represent the Heavy strand (H strand) whereas the black strand represents the light strand (L strand) with their asymmetric gene arrangement. Genes coding for protein and rRNA are interspersed with 22 tRNAs (red bars represented with amino acid code). Genes encoded by the H strand are marked outside whereas genes encoded by the L strand are marked inside by their gene symbols. O_H: origin of replication for H-strand; O_L: Origin of replication of L strand; HSP1: H-strand promoter 1; HSP2: H-strand promoter 2; LSP: L-strand promoter (Scarpulla 2008)

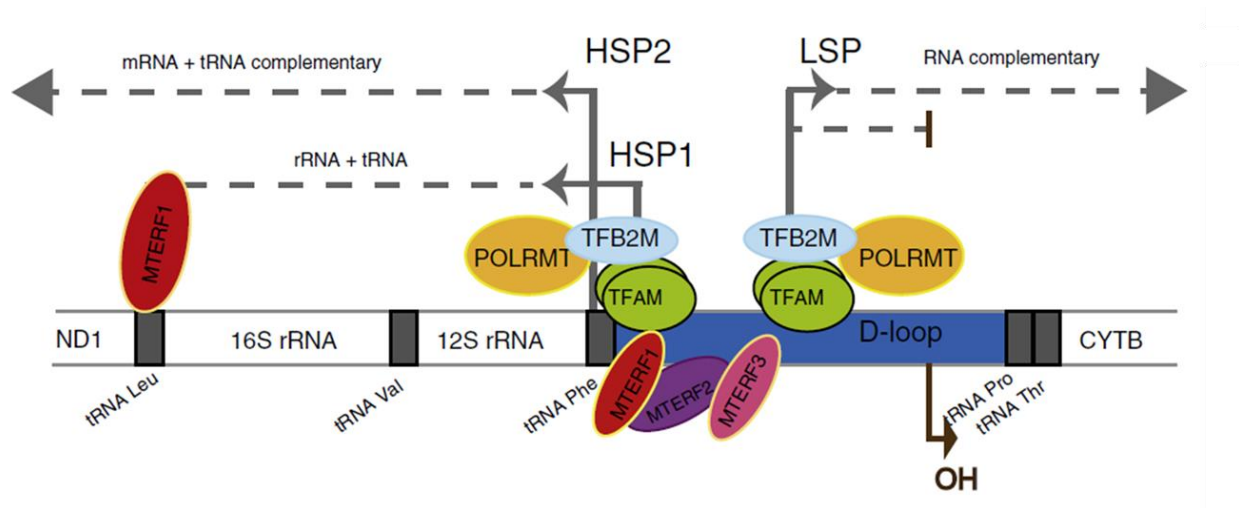


Figure 2: Initiation of Mitochondrial transcription from D-loop: Bidirectional transcription occurs from the H-strand promoters (HSP-1 and HSP-2) and L-strand promoter (LSP) that are located in the D-Loop. HSPs and LSP are bound by TFAM, POLRMT and TFB2M for initiation of transcription. Transcription is terminated by the binding of mitochondrial Termination factors (MTERF1, MTERF2 and MTERF3) to mtDNA (see text for details). OH: origin of replication for H-strand. (Peralta, Wang et al. 2012)

Table 1: mtDNA mutations in mitochondrial diseases

Mutation	MtDNA-encoded gene	Symptoms or disease	Mechanism (if known)	Reference
A1555G	12S rRNA	Deafness due to matrilineal disposition as well as aminoglycoside-induced ototoxicity	Defects in the secondary structure of 12S rRNA	(Matthijs, Claes et al. 1996, Pandya, Xia et al. 1997, Ballana, Morales et al. 2006)
G8529A	MT-ATP8	Reduces the activity of Complex V	Defective assembly of ATP synthase	(Jonckheere, Hogveen et al. 2008)
T8993G	ATPase-6	Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP) syndrome, and the maternally inherited Leigh syndrome (MILS)	Changes Leu-156 with Arg	(Uziel, Moroni et al. 1997)
G11778A	MT-ND4	Leber's hereditary optic neuropathy (LHON)		(Man, Turnbull et al. 2002)
G3460A	MT-ND1			
T14484C	MT-ND6			
A3243G	MT-TL1 (GRNALeu ^{GUAUUU})	Multisystem disorder called MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes) with symptoms such as sensorineural hearing loss, diabetes mellitus, stroke-like episodes, migraine headaches and cardiac problems		(DiMauro and Hirano 1993)

CHAPTER II: DETECTION OF METHYLATION IN MITOCHONDRIAL DNA USING
HPLC-MS/MS IN SH-SY5Y NEUROBLASTOMA CELLS

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2.1 ABSTRACT

Nuclear DNA methylation plays an important role in regulating gene expression. However, presence of mitochondrial DNA (mtDNA) methylation has been debatable for the past four decades due to limitation of the approaches used. In the current study, we used a direct, sensitive and highly specific method using HPLC-ESI-MS/MS to investigate the presence of methylation at global levels in mtDNA in SH-SY5Y neuroblastoma cell line. Differential or incomplete enzymatic digestion of DNA is the pitfall of quantifying DNA methylation by HPLC-ESI-MS/MS. To address this issue, oligonucleotides harboring known amounts of 2'-deoxy-Cytosine (dC) and 5-methyl-2'-deoxy-Cytosine (5mdC) were used as calibrators. 5mdC was detected in a linear and quantifiable manner, along with other nucleosides, using 0.04 µg to 0.8 µg of SH-SY5Y mtDNA with one-step digestion process. Around 3% methylation was found to be present in mtDNA obtained from crude mitochondrial isolates. Similar percentages of methylation were observed even in mtDNA obtained from gradient-purified mitochondria. Purity of mtDNA was characterized indicating elimination of 99.12% nuclear DNA contamination with 115 fold enrichment of mtDNA in gradient-purified mitochondria. Further, linear regression analysis between estimated percentage of mtDNA in various mitochondrial preparations and the corresponding percentage methylation revealed that mtDNA methylation occurs at levels at least as much as that of the nucleus. Presence of methylation in SH-SY5Y mtDNA was further substantiated using methylation-specific PCR for a specific site in 12S rRNA. In conclusion, we established a direct method to quantify global methylation in mtDNA using HPLC-ESI-MS/MS along with addressing issues of variable DNA digestion and the possible chances of nuclear DNA contamination. Further, we reported for the first time, presence of methylated DNA in purified SH-SY5Y mitochondria.

2.2 INTRODUCTION

Mitochondria contain their own genetic information in the form of mitochondrial DNA (mtDNA), which coupled with nuclear genome encode the proteins that form the mitochondria. Alterations in expression of mtDNA-encoded genes can regulate mitochondrial protein expression and eventually affect mitochondrial function. Methylation is known to occur in nuclear DNA, which is catalyzed by a group of enzymes called DNA Methyl-Transferases (DNMTs) (Bestor 2000, Jones 2012). While nuclear DNA methylation and its role in regulating nuclear gene expression are well established (Jones 2012), presence of mtDNA methylation has remained debatable (Maekawa, Taniguchi et al. 2004, Ferreira, Serafim et al. 2015). Although mtDNA methylation was seen as early as 1970s (Vanyushin, Kiryanov et al. 1971, Nass 1973, Vanyushin and Kirnos 1974, Vanyushin and Kirnos 1977, Shmookler Reis and Goldstein 1983, Pollack, Kasir et al. 1984), possible role of mtDNA methylation in regulating the expression of mtDNA-encoded genes was overlooked as no DNA methyl transferases were known to be present in mitochondria and considering the fact that mtDNA lack histones. Recent reports of presence of DNMTs in mitochondria (Chestnut, Chang et al. 2011, Shock, Thakkar et al. 2011, Bellizzi, D'Aquila et al. 2013, Wong, Gertz et al. 2013) and the possible histone-like role of TFAM (Kanki, Nakayama et al. 2004, Shutt, Lodeiro et al. 2010, Lodeiro, Uchida et al. 2012) has rejuvenated interest in studying mtDNA methylation and its potential epigenetic role (Iacobazzi, Castegna et al. 2013, Maresca, Zaffagnini et al. 2015, van der Wijst and Rots 2015).

Although numerous studies have been conducted recently to study mtDNA methylation, conclusions regarding the existence of methylation in mtDNA are dubious with many reports claiming presence of mtDNA methylation (Nass 1973, Chestnut, Chang et al. 2011, Infantino,

Castegna et al. 2011, Shock, Thakkar et al. 2011, Chen, Dzitoyeva et al. 2012, Dzitoyeva, Chen et al. 2012, Feng, Xiong et al. 2012, Bellizzi, D'Aquila et al. 2013, Byun, Panni et al. 2013, Jia, Li et al. 2013, Pirola, Gianotti et al. 2013, Wong, Gertz et al. 2013, Ghosh, Sengupta et al. 2014, Baccarelli and Byun 2015, Janssen, Byun et al. 2015, Zheng, Linarelli et al. 2015) while few other studies reporting it to be absent (Dawid 1974, Groot and Kroon 1979, Maekawa, Taniguchi et al. 2004, Hong, Okitsu et al. 2013). Technical limitations and inability to reproduce results by various approaches used for studying mtDNA methylation have hindered in dispelling the doubts about occurrence of mtDNA methylation (Dahl and Guldberg 2003, van der Wijst and Rots 2015). MtDNA methylation has been detected by Methylation Sensitive Restriction Enzyme Analysis (MSRE) followed by Southern-Blot hybridization using radio-labelled probes (Nass 1973, Vanyushin and Kirnos 1974, Vanyushin and Kirnos 1977, Shmookler Reis and Goldstein 1983, Pollack, Kasir et al. 1984), Methylated-DNA-IP (Shock, Thakkar et al. 2011, Bellizzi, D'Aquila et al. 2013, Ghosh, Sengupta et al. 2014), 5mC-ELISA (Dzitoyeva, Chen et al. 2012), methylation-specific PCR (Pirola, Gianotti et al. 2013) and Bisulfite-sequencing (Bellizzi, D'Aquila et al. 2013, Wong, Gertz et al. 2013, Ghosh, Sengupta et al. 2014). Nuclear DNA contamination is the common pitfall for all these approaches and needs to be taken into account. MSRE limits the study of mtDNA methylation at sequence-specific sites whereas reproducibility using different antibodies for methylated-DNA-IP and 5-mC-ELISA has been questioned (Maresca, Zaffagnini et al. 2015). Even though bisulfite-sequencing is considered the gold-standard for measuring DNA methylation and has been the most utilized approach for determining DNA methylation, incomplete bisulfite-conversion, bias in PCR-amplification, preferential cloning, low coverage considered for analysis and the inability to distinguish 5-mdC from other modified cytosines (5-hydroxyl-methyl-deoxy-cytosine - another epigenetic

modification present in mtDNA (Shock, Thakkar et al. 2011, Dzitoyeva, Chen et al. 2012)) have plagued the results.

HPLC-MS/MS is by far the most-direct, specific and sensitive method to quantify global levels of 5-mdC in the nuclear DNA but does not provide site-specific information. Variability in DNA digestion efficiency and nuclear DNA contamination can affect accuracy and reproducibility of detection of DNA methylation in mitochondria using this approach (van der Wijst and Rots 2015). Although a previous study reported use of HPLC-MS/MS for detection of mtDNA methylation (Infantino, Castegna et al. 2011), these issues regarding DNA digestion and nuclear contamination were either overlooked or not addressed at depth that should be accounted for reliable quantification.

In the current study, we established a direct and specific method to quantify global methylation in mtDNA using HPLC-MS/MS. We addressed the issue of variability during digestion of DNA by using custom oligonucleotides as controls. We further reported presence of methylated DNA in percoll-gradient purified SH-SY5Y mitochondria. To address the issue of nuclear DNA contamination, we have characterized purity of our mtDNA preparations. Additionally, we have assayed mtDNA methylation at a specific site in 12S rRNA by methylation-specific primer.

2.3 MATERIALS AND METHODS

Cell Culture and its harvest

SH-SY5Y neuroblastoma cells were cultured in DMEM containing 25 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), 1% Pen-strep and maintained at 37°C, 5% CO₂. Cell lines

growing in the culture flasks or petri-dishes were rinsed with 1X Phosphate Buffered Saline (PBS), trypsinized and then harvested using the growth media. The harvested cells were then pelleted at 800g for 5 minutes at 4°C and subsequently washed with 1X PBS at 4°C for subsequent downstream procedure.

Isolation of crude and percoll-gradient purified mitochondria

Crude preparation of mitochondria: Mitochondria were isolated as described previously (Kristian, Hopkins et al. 2006) with some modifications. Briefly, cells growing in a confluent T180 flask were harvested as described above. The washed cell pellets were then re-suspended in Mannitol-Sucrose-HEPES-EGTA (MSHE) buffer, collected in a pre-cooled cavitation chamber (nitrogen bomb; Parr Instrument Company, Moline, IL) and subjected to 900 psi for 15 minutes. The cell suspension is collected by releasing the pressure instantaneously and centrifuged at 1000g for 5 minutes at 4°C to pellet the cell debris. The supernatant was collected and spun at high speed of 20000g for 10 minutes at 4°C. The pellet obtained was re-suspended in MSHE and is the crude preparation of mitochondria.

Percoll gradient-purified mitochondria: Gradient-purified mitochondria were isolated in a modified version as described previously (Kristian, Hopkins et al. 2006). Percoll was purchased from GE-Healthcare (catalogue # 28-9038-48) and 100% percoll was prepared by mixing 9 parts of Percoll with 1 part of 1.5M NaCl. The crude pellet of mitochondria obtained is re-suspended in 800 µL of 15% percoll and layered on to a gradient of 50% and 21% percoll. The resulting percoll-gradient underwent ultra-centrifugation in a swing-bucket rotor system (SW 28.1 rotor) at 30,700 g for 15 minutes at 4°C. The pure mitochondria separated between 50 and 21% percoll,

was collected, diluted with MSHE and then washed at 20,000 g for 15 minutes at 4°C twice. The pellet obtained is the percoll-gradient purified mitochondria.

Mitochondrial lysate of crude and gradient-purified mitochondria were prepared using M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Protein concentrations for the lysates were determined using a BCA Protein Assay kit (BioRad).

DNA extraction

DNA extraction was done by the method based on organic-solvent extraction of DNA as described previously (Guo, Jiang et al. 2009). Briefly, the sample in ice-cold lysis buffer underwent dounce homogenization, followed by Proteinase K digestion at 55°C for 3h in a water bath. The mixture was spun to remove the non-soluble fractions and the DNA in the supernatant was extracted by standard Phenol-Chloroform-Isoamyl alcohol method. DNA was precipitated using 3M Sodium Acetate and isopropanol at -20°C. The DNA pellet was washed twice with 70% ethanol. Finally, the extracted DNA pellet is then air-dried, re-suspended in nuclease-free water and then quantitated using Infinite M200 (Tecan, San Jose, USA).

SH-SY5Y mtDNA extraction: The mitochondrial DNA was extracted from both the crude and gradient-purified mitochondria for determining the amount of mtDNA methylation. Twenty million cells in a T180 flask were seeded and harvested 48 hours later for extraction of mitochondria as described above. The isolated mitochondria were suspended in ice-cold Lysis buffer for DNA extraction as described above.

Total DNA extraction: SH-SY5Y cells were harvested 48 hours after seeding as described above and the washed cell pellets were suspended in ice-cold Lysis buffer for DNA extraction as described above.

Long PCR of human mtDNA

SH-SY5Y mtDNA was extracted and used as template to generate a PCR product of 15.745 Kb long to represent the un-methylated version of human mtDNA as described previously (Binder, Dunn et al. 2005) using Expand Long Range, dNTPack (Roche Diagnostics). The PCR product thus obtained was purified using Qiaquick PCR purification kit (Qiagen) before using it for HPLC-ESI-MS/MS assay, following digestion and for methylation-specific PCR assay, following bisulfite-conversion.

Digestion of DNA

Oligo-deoxy-nucleotides, mtDNA, total DNA, Long PCR were digested as described previously (Quinlivan and Gregory 2008). Briefly, known amount of the DNA of interest was digested in 100 μ L reaction volume, using the enzymes- 2.5 U Benzonase (Sigma E1014), 3 mU Phosphodiesterase I (Sigma P3243) and 2U Alkaline Phosphatase (Sigma P7923) in Tris-HCl (20 mM. pH 7.9) containing 1 mM NaCl and 0.2 mM $MgCl_2$ at 37°C for 20-24 hours.

HPLC-ESI-MS/MS

Reagents: 5-Methyl-2'-deoxycytidine (5MdC) was purchased from Berry & Associates, Dexter, MI (Catalogue# PY7635). The four other deoxyribonucleosides- 2'-deoxycytosine (dC), 2'-

deoxyguanosine (dG), 2'-deoxyadenosine (dA), and 2'-deoxythymidine (dT), were purchased from Sigma Chemical Co. (St. Louis, MO).

Apparatus: Waters Acquity "Classic" (Waters Corp. Milford, MA) UPLC system was used with an Altima C18 column that had internal diameter of 2.1 mm and length of 50 mm, with sorbent particles of size 3.5 μm (Grace, Columbia, MD). Triple quadrupole mass-spectrometer (Quattro Ultima Micromass Ltd. Manchester, UK) was coupled with the UPLC system through electron-spray probe for detection and quantification of nucleosides.

LC-ESI-MS/MS Procedure: Released nucleosides in the digestion buffer of a particular sample of interest were separated by UPLC for MS/MS detection, adapting a general strategy used for separating a complex mixture of nucleosides. The injection volume used was 20 μL . LC separation was performed at a flow rate of 300 $\mu\text{L}/\text{minute}$. Two buffers, A: 10 mM ammonium acetate (NH_4OAc) and B: 10% Methanol comprised the mobile phase that was used for elution of the nucleosides using the following gradient:

Time (min)	A%	B%
0.0	99	1
1	99	1
2	90	10
9	20	80
11	99	1

The mobile phase effluent were passed through the electrospray probe (source block temperature: 110°C, de-solvation temperature: 200°C and cone voltage: 50V) and tandem mass spectra was acquired on triple quadrupole in multiple reaction monitoring (MRM) mode. The

first quadrupole (*Q1*) that acts as a mass-filter, allowed a parent ion of a specific mass-to-charge ratio to enter the second quadrupole. The second quadrupole (*Q2*) is the collision cell, where Argon gas at a pressure of 2×10^{-3} mbar was used to fragment the ions through the process of collision-activated disassociation (collision energy: 15 eV). The resulting fragment ions were transmitted to the third quadrupole (*Q3*), where only the daughter ions of a desired mass-to-charge ratio were allowed to pass and reach the detector for further quantification to compose the tandem mass spectra. Selected ion recordings for 5-Methyl-2'-deoxycytidine (5MdC) was 242.1 (parent ion) >126.1 (daughter ion) and for the other nucleosides were dC: 229.1>112.1, dT: 244.1>127.1, dA: 253.1>136.1, dG: 269.1>152.1 with dwell time of 0.1 second. To monitor instrument performance, every batch of samples were punctuated with duplicate preparations of 5MdC standard curve, ranging from 1 to 20 pmoles in a mixture of 40 pmoles of each of the nucleosides dA, dT, dG and dC in 10 mM NH_4OAc as well as only 10mM NH_4OAc as the blank.

Determination of mtDNA purity using qPCR

Whole-cell DNA, mtDNA from crude and gradient-purified mtDNA were extracted from equal number of cells and the DNA was re-suspended in equal volume of nuclease-free water. Equal amount of DNA from each of them were used to determine the amount of nuclear-encoded and mitochondrial-encoded genes -*18S rRNA* and *16S rRNA*, respectively- by real-time PCR. Fold-enrichment of mtDNA was calculated by comparing ratio of *16S rRNA* to *18S rRNA* in crude and gradient-purified mtDNA with respect to whole-cell DNA. *18S rRNA* /*16S rRNA* was then used to determine the percentage of nuclear DNA eliminated in crude and gradient-purified mtDNA with respect to whole-cell DNA similar to as reported previously (Wilkins, Carl et al. 2015) and corrected to account for change in proportion of mtDNA with purification.

An estimate of fraction of bases of mtDNA present in each sample of DNA preparation with respect to nuclear bases was derived from the percentage of nuclear DNA eliminated and mtDNA enrichment, wherein the ratio of number of bases in the mtDNA to nuclear DNA (33138 bases in one copy of mtDNA and 1.28×10^{10} bases in one copy of nuclear DNA) and also the mtDNA copy number per copy of nuclear DNA has been accounted for. The estimated amount of mtDNA in each sample thus calculated was represented as a percentage of mtDNA present with respect to total DNA (sum of nuclear and mtDNA).

Linear regression analysis of percentage methylation and percentage mtDNA

Simple linear regression analysis was performed between percentages of mtDNA in whole-cell DNA and mtDNA extracted from mitochondrial preparations (with increasing purity levels) with its respective percentage methylation in order to investigate whether there is a statistically significant relationship between the two parameters – percentage mtDNA and percentage methylation. Following linear regression model was used, where β is the coefficient that specifies the increase in methylation based on the increase in percent mtDNA, and ϵ is an error term: Methylation = (Percent mtDNA)* β + ϵ

Bisulfite conversion and Methylation-specific-PCR assay

Bisulfite conversion: The extracted mtDNA of interest and ‘Long PCR’ was treated with bisulfite using EZ DNA methylation-Gold kit (Zymoresearch, Irvine, CA, USA). Bisulfite sulphonates and then deaminates the un-methylated Cytosine to produce uracil in DNA. Thus methylated cytosines are protected from the conversion to uracil, allowing the use of site-specific PCR amplification i.e. methylation-specific PCR. Briefly, 350 ng of mtDNA in 20 μ L nuclease-free

water was mixed with 130 µL of CT Conversion reagent and was placed in the thermal cycler where the program consisted of initial denaturation at 98°C for 10 minutes, followed by conversion at 64°C for 2.5 hours. The converted solution of mtDNA was mixed with binding buffer to allow its binding to the column. A high speed spin was followed by washes, desulphonation for 20 minutes at room temperature and then finally eluted using the Elution buffer to obtain the bisulfite-treated DNA.

After obtaining the converted mtDNA, the following primers were used to amplify a region of 12S rRNA using the following PCR program. The primers were designed using the bisulfite treated version of the Cambridge reference sequence of human mtDNA (Anderson, Bankier et al. 1981).

F: AGTTATAAGTTTAAAATTTAAAGGATTTGGC

R: ACTTAAAAAAAATAACGAACGATATATACGCG

PCR set up: .4 µM of the forward and reverse primer was used to amplify to obtain a 320 bp amplicon using ZymoTaq Premix (Zymoresearch, Irvine, CA, USA).

PCR program: Initial denaturation of 10 mins at 95°C was followed by 30 cycles of denaturation at 95 °C for 30 secs, annealing at 51.5°C for 45 secs and synthesis at 72°C at 45 secs, with a final elongation step of 7 mins at 72°C.

Agarose-gel-electrophoresis: The subsequent PCR products obtained were electrophoresed through 1% agarose gel containing 0.5 µg/ml ethidium-bromide and visualized under ultraviolet light using a Bio-Rad ChemiDoc Imager.

Immunoblotting

Whole cell lysates were prepared by M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Protein concentrations for all the lysates were determined using a BCA Protein Assay kit (BioRad).

Equal amount of proteins from the protein lysates were resolved by electrophoresis in pre-cast 4–12% gels (BioRad), and then transferred to polyvinyl di-fluoride (PVDF) membranes. Non-specific binding was blocked by gently agitating the membranes in 5% non-fat milk and 0.1% Tween in PBS (PBST) for 1 hour at room temperature. Blots were subsequently incubated in 5% bovine serum albumin (BSA) containing 0.1% Tween with the designated primary antibody overnight at 4°C with gentle agitation. After PBST washes, the blot was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature with gentle agitation. After PBST washes, the blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Chemiluminescence signals were detected using a Bio-Rad ChemiDoc Imager.

Primary antibodies for, VDAC, β -Tubulin, GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA) and that of HDAC1 was purchased from Pierce (Thermo Scientific, Eugene, OR, USA). All of the antibodies were used at a 1:1000 dilution.

Data analysis

Data are expressed as means \pm standard error of the means (SEM). To compare means between two groups we used two-way, unpaired Student's t-tests, with p-values of <0.05 considered significant.

2.4 RESULTS

Characterization of 5mdC along with dA, dG, dT and dC by HPLC-ESI-MS/MS

HPLC-ESI-MS/MS was used to characterize and quantify 5mdC along with dA, dG, dT and dC. Standards of each nucleoside were used for initial characterization by mass spectrometry. Electron-spray-ionization (ESI) generated ions obtained at m/z 253.1, 244.1, 269.1, 229.1 and 242.1 corresponded to protonated nucleosides for dA, dG, dT, dC and 5mdC, respectively. The collision-assisted dissociation (CAD) of these ions resulted in generation of daughter fragments. Most abundant daughter ions were obtained at m/z 136.1, 127.1, 152.1, 112.1 and 126.1 corresponding to the protonated bases (A, G, T, C, 5mC) resulting from glycosidic-bond cleavage of the respective protonated-nucleosides. Thus, the unique MRM transitions obtained at m/z 253.1>136.1, 244.1>127.1, 269.1>152.1, 229.1>112.1 and 242.1>126.1 were followed to detect and quantify dA, dG, dT, dC and 5mdC simultaneously as depicted in **Figure 1A**. Under optimized elution and ESI conditions, we were able to resolve peaks corresponding to dA to A, dG to G, dT to T, dC to C and 5mdC to 5mC transitions with typical chromatogram shown in **Figure 1B and D**.

Using equimolar mixture of dA, dG, dT and dC ranging from 10 to 100 pmoles prepared in 10 mM Ammonium acetate, we were able to linearly detect these nucleosides simultaneously. The corresponding linear equations (representative) with coefficient of regression are shown in **Figure 1C**. Further, various concentrations of 5mdC were spiked in equimolar mixture of dA, dG, dT and dC (40 pmoles each) for quantification of 5mdC. We were able to detect 5mdC linearly in range of 1 to 20 pmoles as shown in **Figure 1E** by representative curve with correlation coefficient of 0.9925. The measured 5mdC levels using this external calibration curve

were found to be in proportion with nominal 5mdC levels as shown in **Figure 1F**. Further, limit of detection of 5mdC under the experimental conditions was found to be 25 fmoles as shown in **Figure 1D**. All these external calibration curves were included in every run for quality control.

Linear digestion and quantification of nucleosides in oligodeoxynucleotides containing methylated and un-methylated cytosine

Inefficient and variable digestion of DNA samples into nucleosides is a major limitation for accurate quantification of nucleosides from DNA samples using HPLC-MS/MS. Since we wanted to simulate the digestion conditions in real DNA samples, we used custom-made oligo-deoxy-nucleotides either containing only dC or dC in combination 5mdC to investigate if we could obtain linear digestion of these oligo-deoxy-nucleotides and further quantify resulting nucleosides in linear manner. We obtained linear and proportional detection of dA, dG, dT and dC when un-methylated oligo-deoxy-nucleotide (ranging from 0.2 to 4 µg DNA in 100 µl of digestion mix) was digested and analyzed. The corresponding DNA amounts actually injected and analyzed ranged from 0.04 to 0.8 µg based on 20 µl injection volume. Representative curves, linear equations, and coefficient of regression along with nominal amount of respective nucleoside present in oligo-deoxy-nucleotide are shown in **Figure 2A**. Digestion was linear up to 5 µg of DNA (in 100 µl of digestion mix) and was saturated beyond this amount of DNA for the given digestion conditions (data not shown). 5mdC was not detected in un-methylated oligo-deoxy-nucleotide digested product confirming specificity of our method. Similarly, oligo-deoxy-nucleotide containing both dC and 5mdC (with similar range of DNA amounts as used for un-methylated oligo-deoxy-nucleotide) was digested and analyzed. Linear and proportional detection of all the nucleosides (dA, dG, dT, dC and 5mdC) were obtained in the studied range

suggesting linear digestion of all nucleosides under optimized digestion conditions. Representative curves, linear equations, and coefficient of regression along with nominal amount of respective nucleoside present in oligo-deoxy-nucleotide samples are shown in **Figures 2B** and **2C**. Further, measured 5mdC levels using this oligo-deoxy-nucleotide calibration curve were found to be in proportion with nominal 5mdC levels as shown in **Figure 2D**. Calibration curves obtained by using digestion product of 5mdC containing oligo-deoxy-nucleotide was used to calculate the amount of 5mdC in test samples instead of using calibration curve obtained by analytical grade 5mdC in equimolar mixture of other nucleosides. This was done to account for any variability introduced during digestion process and is more comparable to conditions in test samples. Further, our calibration curves covered concentration range of each nucleoside expected to be present in test DNA samples. Both un-methylated and methylated oligo-deoxy-nucleotide standards were included in each run.

Linear and proportional detection of nucleosides in SH-SY5Y mtDNA

Since we wanted to measure methylation in mtDNA, we first investigated, using our digestion conditions for custom oligodeoxynucleotides, if we could obtain linear digestion of mtDNA and quantify resulting nucleosides. MtDNA was obtained from mitochondria isolated from SH-SY5Y cells and digested (0.2 to 4 μ g mtDNA was used for digestion in 100 μ l of digestion mix) as described previously **Figure 3A**. MtDNA displayed a single band on 1% agarose gel which resulted into smear upon digestion as shown in **Figure 3B**. We obtained linear detection of dA, dG, dT, dC and 5mdC in SH-SY5Y mtDNA (DNA analyzed ranged from 0.04 to 0.8 μ g based on 20 μ l injection volume). Representative curves, linear equations, coefficient of regression along with calculated amount of respective nucleosides present in SH-SY5Y mtDNA are shown

in **Figure 3C-G**. Further, calculated amount of nucleosides increased in proportion to the amount of DNA analyzed. Similar to oligo-deoxy-nucleotide experiments, digestion was linear up to 5 μ g of DNA (in 100 μ l of digestion mix) and was saturated beyond this concentration (data not shown). 2 μ g of mtDNA (in 100 μ l of digestion mix) was used for digestion in all the following experiments for analyses, which is within the linear range of digestion of mtDNA.

Detection of 5mdC in mtDNA extracted from crude and gradient-purified mitochondria

As we were able to linearly digest and quantitate all nucleosides including 5mdC in DNA obtained from isolated mitochondria, we wanted to quantitate and confirm presence of 5mdC in SH-SY5Y mitochondria isolated using different methodologies (which are conventionally used to get pure mitochondria) and prepared at different times over an extended time period of about a year (n=5-6). We determined absolute values of 5mdC (pmoles/ μ g DNA) in ‘Long PCR’, whole-cell DNA, mtDNA isolated from crude and gradient-purified mitochondria as depicted in **Figure 4A**. As the name suggests, ‘Long PCR’ denotes the PCR fragment generated from SH-SY5Y mtDNA of 15,745 base-pair in size, almost the size of human mtDNA as described previously (Binder, Dunn et al. 2005). As ‘Long –PCR’ of mtDNA has been generated by DNA Polymerase that does not copy the methylation like DNMT1, it is devoid of any methylation and thus serves as a negative-control for our HPLC-ESI-MS/MS assay. Indeed, no 5mdC was detected in ‘Long-PCR’ of mtDNA, thus further confirming the specificity of our assay. Nuclear DNA methylation is well established (Bestor 2000, Jones 2012) and 5mdC was successfully detected in whole-cell DNA. Further, 5mdC was detected in mtDNA isolated from both crude mitochondria and gradient-purified mitochondria and moreover, were not significantly different from each other.

To account for inter-sample variation due to the possibility of differential enzymatic digestion, sample preparation and downstream analysis, the absolute values of 5mdC were normalized by the amount of dC and the sum of 5mdC and dC, and subsequently represented in terms of percentages as shown in **Figure 4B-C**. 5mdC percentages were found to be comparable between whole-cell DNA and mtDNA extracted from crude and gradient-purified mitochondria, approximately 3% in each of them. These results suggest that mtDNA has similar percentage methylation as the nuclear DNA in SH-SY5Y cells. Our results for percentage methylation are in agreement with that reported for both the nuclear DNA (Laurent, Wong et al. 2010) and mtDNA (Pollack, Kasir et al. 1984, Rebelo, Williams et al. 2009, Byun, Panni et al. 2013) in literature and remain quite consistent in all our other experiments conducted using SH-SY5Y cell line assuring our methodology to be reliable.

Purity characterization of mtDNA extracted from crude and gradient-purified mitochondria

Next, we characterized purity of our mitochondrial preparations in comparison to the whole-cell lysates. The purity of the preparation of mitochondria at protein level was determined by the presence of VDAC (mitochondrial-marker), HDAC1 (nuclear-marker) and β -Tubulin (cytosolic-marker) as shown in **Figure 5A**. Both the crude and gradient-purified preparations of mitochondria were highly enriched in mitochondrial content as observed by the remarkable increase of VDAC levels in them compared to whole cell lysate. Minimal nuclear contamination was found in the crude mitochondrial preparations, whereas gradient-purified mitochondria did not have any detectable nuclear contamination, as observed by the presence of HDAC1 in them. Further, cytosolic contamination was minimal in the crude preparation of mitochondria and not detectable in gradient-purified mitochondria in comparison to the whole-cell lysates. These

results indicate that the crude and gradient-purified mitochondria are free of nuclear contamination at protein level considerably.

MtDNA extracted from crude-preparation of mitochondria was seen as a single band, with no apparent smear, thus suggesting the absence of nuclear DNA (**Figure 3B**). However, we wanted to assess nuclear DNA contamination in a quantitative way and not just qualitatively as studied previously (Infantino, Castegna et al. 2011). In order to address the possibility of nuclear DNA contamination in mtDNA isolated from crude and gradient-purified mitochondria, qPCR-based method was used to determine the levels of mtDNA and nuclear DNA by amplifying 18S rRNA and 16S rRNA. We report that 97.5% of nuclear DNA was excluded from mtDNA isolated from the crude preparation of mitochondria and mtDNA obtained in such a manner, was enriched by 45 fold (as determined by measuring 16S/18S ratio) in comparison to the whole-cell DNA, whereas 99.1% of nuclear DNA was eliminated from mtDNA isolated from the gradient-purified preparation of mitochondria and was enriched by 115 fold in comparison to the whole-cell DNA as depicted in **Figure 5B-C**.

Linear regression analysis of percentage methylation and percentage mtDNA in mitochondrial preparations suggests presence of mtDNA methylation

Considering the substantial difference in the size between nuclear genome and mitochondrial genome (33138 bases in mtDNA and 1.28×10^{10} bases in nuclear DNA), the small fraction of nuclear DNA remaining in mitochondrial DNA preparations (0.88% in gradient purified mitochondria with respect to whole-cell), can potentially cause significant contribution to the detected 5-methyl cytosine and thus, confound our analysis. In contrary to the small size of mitochondrial genome with respect to that of the nuclear genome, mtDNA is present in

numerous copies (thousands) per copy of nuclear genome which should also be considered in analysis. Thus, we adopted a conservative approach to estimate fraction of bases of mtDNA present with respect to nuclear bases in each sample, from the percentage of nuclear DNA eliminated and the fold-enrichment of mtDNA. We have also accounted for the ratio of number of bases in the mtDNA to that of nuclear DNA as well as mtDNA copy number w.r.t the copy of nuclear DNA in each sample of DNA preparation (whole-cell DNA, crude and gradient-purified mtDNA). The estimated percentages of mtDNA obtained were found to be $0.6\% \pm 0.13$, $21\% \pm 4.06$ and $41\% \pm 5.06$ for whole-cell DNA, crude mtDNA and gradient-purified mtDNA, respectively.

Next, a linear regression analysis of percentage of mtDNA in a sample of DNA (with increasing purity levels of mtDNA) with its respective percentage methylation was performed to determine whether mtDNA methylation contributes significantly to overall observed methylation levels. Analysis was performed using triplicates for whole-cell DNA, crude and gradient-purified mtDNA signifying levels of increasing purity. Assuming the arbitrary rates of methylation for mtDNA and nuclear DNA as M and N respectively, the four possible scenarios of regression models are depicted in **Figure 6A-D**. **Figure 6A** represents the scenario assuming M to be 0 (i.e. no mtDNA methylation) with a negative slope ($\beta < 0$). **Figure 6B** represents a similar scenario of negative slope but where mtDNA methylates at a level greater than 0 but lesser than that of nuclear DNA (i.e. $M > 0$ and $M < N$). **Figure 6C** represents the scenario where mtDNA methylates at a rate equal to that of nuclear DNA ($M = N$). **Figure 6D** represents the scenario where the rate of mtDNA methylation is greater than that of the nuclear DNA and where β would be greater than zero (positive slope). Our simple linear regression analysis revealed a significant correlation between percentage mtDNA and percentage methylation levels corresponding to the scenario as

depicted in **Figure 6D** with a p value of 0.00879 and coefficient of regression $\beta = 3.333$, with 95% confidence interval of (0.780, 5.885) which does not include 0, as shown in **Figure 6E**. This suggests that mtDNA methylation occurs at an observable level at least as much as methylation for nuclear DNA.

MtDNA methylation observed by methylation-specific PCR at a site-specific level

As we observed presence of methylation at global levels in DNA obtained from purified-mitochondria of SH-SY5Y cells, we wanted to confirm our findings using an alternate strategy. Therefore, we investigated methylation in mtDNA at site-specific level using methylation-specific PCR. MtDNA extracted from isolated mitochondria was treated with bisulfite leading to sulfonation of mtDNA which converts the unmodified cytosines to uracil whereas modified cytosines remain the same as bisulfite-treatment cannot de-amine them by sulphonation. Thus, by using primers that specifically would amplify a region of mtDNA containing the unchanged cytosine in bisulfite-treated DNA (actually a modified C in the original DNA), presence of methylated cytosines can be detected by methylation-specific PCR as diagrammatically represented in **Figure 7A**.

There are 435 CpG sites in the Cambridge reference sequence of human mtDNA. By using the MethPrimer software (Li and Dahiya 2002) for prediction of CpG islands according to the criteria:- Island size > 100 bp, GC Percent > 30.0, Observed/Expected > 0.6, 44 CpG islands were found in human mtDNA. We selected a CpG island in 12s rRNA for further site specific study. 12s rRNA has been previously studied by several groups where mtDNA methylation has been determined (Shock, Thakkar et al. 2011, Byun, Panni et al. 2013, Iacobazzi, Castegna et al. 2013) and its functional association is shown (Byun, Panni et al. 2013). We designed

methylation-specific primers for a specific site in selected CpG island of 12s rRNA as per the recommendations published previously (Li 2007). A single band of PCR product indicated presence of methylation at this site as shown in **Figure 7B**. Additionally as negative-controls, we used 'Long PCR of mtDNA' as the template under the same PCR conditions that did not amplify a band, similar to the 'no template' PCR reaction indicating specificity of our primers and thus, our method.

2.5 DISCUSSION

The status of mtDNA methylation has remained questionable since 1970's due to limitations of the approaches used (van der Wijst and Rots 2015). Nevertheless, involvement of mtDNA methylation in regulating transcription of mtDNA-encoded genes and subsequently mitochondrial function is being contemplated (Chestnut, Chang et al. 2011, Shock, Thakkar et al. 2011, Bellizzi, D'Aquila et al. 2013, Iacobazzi, Castegna et al. 2013, Pirola, Gianotti et al. 2013, Wong, Gertz et al. 2013). In this study, a highly specific and direct method of HPLC-ESI-MS/MS was established to determine the presence and quantify global mtDNA methylation in SH-SY5Y neuroblastoma cells, along with addressing the issues regarding nuclear DNA contamination and the possibility of differential enzymatic digestion of DNA. We have for the first time reported presence of methylation, at global level, in human mtDNA prepared from gradient-purified mitochondria. Further, our results about percentage methylation in SH-SY5Y mtDNA are consistent with that reported previously using different approaches other than HPLC-ESI-MS/MS (Pollack, Kasir et al. 1984, Rebelo, Williams et al. 2009, Byun, Panni et al. 2013). Moreover, existence of methylation in SH-SY5Y mtDNA was further substantiated using methylation-specific PCR for a specific site in 12S rRNA.

Variability in DNA digestion efficiency and possibility of nuclear contamination are the two critical issues that should be addressed carefully for accurate quantification of mtDNA methylation by HPLC-MS (Dahl and Guldberg 2003). Unlike a previous study (Infantino, Castegna et al. 2011) that has determined mtDNA methylation levels by HPLC-MS/MS using external standard curves derived from analytical-grade nucleosides, our study utilized oligo-deoxy-nucleotides with known amounts dC and 5mdC as calibrators, so that the possibility of differential digestion efficiency is accounted in the calibration curve. Thus, our methodology used for calibration is more similar and replicative of the actual process of DNA digestion. Further, previous studies have employed the Crain method for digestion of mtDNA (Crain 1990), which is a three-step digestion process requiring prior denaturation of DNA and adjustments of pH/incubation temperature at different stages. Here we used a much simplified protocol where the nuclease was substituted by Benzonase requiring just one step digestion as described previously (Quinlivan and Gregory 2008), thus reducing the chances of variability introduced during digestion process. Furthermore in our study, we ensured linear and proportional digestion of oligo-deoxy-nucleotides as well as mtDNA and the used concentrations of the test samples were within this range for reliable measurements.

In the current study, the possibility of nuclear DNA contamination was addressed critically and assessed both qualitatively and quantitatively compared to previous study where this issue was poorly reported and addressed only qualitatively using less sensitive approaches (Infantino, Castegna et al. 2011). The purity of our mitochondrial preparations, at protein level, was assessed by determining the presence of nuclear-marker and was found to be significantly free of nuclear contamination. Further, isolated mtDNA displayed a single band on agarose gel suggesting minimal levels of nuclear DNA contamination. Moreover, qPCR-assisted

measurements of nuclear contamination suggested that 99.12% of nuclear DNA was eliminated from mtDNA isolated from the gradient-purified preparation of mitochondria and our mtDNA preparations were highly enriched (up to 115 fold) for mtDNA compared to whole cell DNA. Although we found only 0.88% of nuclear DNA remaining in gradient purified mitochondria w.r.t. whole cell DNA, this can be substantial relative to the amount of mtDNA as mitochondrial DNA comprises only a small fraction (less than 1%) of total cellular DNA (because of its very small size, in spite of being present in numerous copies). The estimated percentages of mtDNA were found to be 0.6%, 21% and 41% for whole-cell DNA, crude mtDNA and gradient-purified mtDNA, respectively after accounting for difference in size of nuclear genome compared to mitochondrial genome and also accounting for the mitochondrial DNA copy number. Thus, the fraction of nuclear DNA contamination was estimated to be substantial relative to the mtDNA present in purified mitochondrial preparations and caution must be placed while interpreting our results. However, linear regression analysis performed between percentages of mtDNA in whole-cell DNA and mtDNA extracted from mitochondrial preparations (with increasing purity levels) with its respective percentage methylation, suggested that mtDNA methylation does contribute significantly to overall methylation and mtDNA methylation occurs at least at levels similar to methylation for nuclear DNA. Although our results suggested significant presence of mtDNA methylation at global levels, additional studies will need to be conducted in future to achieve more purified mtDNA in order to substantiate our findings.

Lack of methods to isolate completely pure mitochondrial DNA is a bottleneck in the field of mitochondrial DNA methylation. A recent study (Jayaprakash, Benson et al. 2015) established a method to isolate mtDNA from whole-cell DNA using repetitive treatments with Exonuclease V to achieve 98% pure mtDNA. As Exonuclease V selectively degrades single stranded DNA in

ATP-dependent manner, the rationale was to eliminate single stranded DNA fragments most likely derived from the nuclear genome, while keeping the circular mtDNA intact. Along similar lines, we attempted purifying mtDNA using repetitive treatments with Exonuclease V followed by column purification. In order to achieve higher purity, we used DNA extracted from isolated mitochondria for Exonuclease V-treatment, instead of just using whole cell DNA, as used in the above mentioned study. Our preliminary data (n =1) suggested unprecedented enrichment of about 1300 fold for mtDNA (compared to 115 fold enrichment in gradient purified mtDNA) using this combinatorial approach of mitochondrial isolation and Exonuclease V treatment. It should be mentioned that crude mitochondrial isolation technique was utilized for this study; thus, we speculate that using gradient purification of mitochondria along with Exonuclease V treatment can further improve the purity of mtDNA preparation. Further studies will need to be conducted in the future in order to examine the reproducibility of this approach followed by analysis of methylation level both at global levels using our established HPLC-MS assay and at specific site using methylation-specific PCR assays.

Apart from studying mtDNA methylation at global levels using HPLC MS/MS, the presence of methylation in SH-SY5Y mtDNA was further substantiated using methylation-specific PCR for a specific site in 12S rRNA. Although, in our study we used long PCR of mtDNA (an unmethylated amplicon of mtDNA) as negative control, the possibility of obtaining methylation from insertions of mitochondrial DNA in the nuclear genome (NUMT) needs to be ruled out by utilizing ρ^0 cell lines that lack functional mtDNA, as a negative control in addition to unmethylated amplicon of mtDNA. In a preliminary study, our methylation-specific primers amplified a product from SH-SY5Y ρ^0 cell line raising potential concern about the specificity of primers for mitochondrial genome. However, this analysis requires further confirmatory studies

as amplification was also observed from un-methylated amplicon of mtDNA in this study (which was not observed in any of our previous studies) raising concern about possible reagent contamination that needs to be resolved and carefully examined in future. Further, this analysis can also be confounded by the fact that, although ρ^0 cell lines cannot form a functional respiratory chain, but they might have fragments of mtDNA from damaged mtDNA. Alternatively, to address specificity of methylation-specific primers, BLAST analysis was performed which revealed 100% match of primers only to the mitochondrial sequence. However, two nuclear hits in chromosome 11 and 17 were also identified, but with less than 85% match and mismatches being in the reverse primer sequence suggesting less likely amplification from these sites. Further, it should be noted that the site-specific analysis was conducted using isolated mitochondria where majority of nuclear DNA contamination is eliminated. Also, in contrast to analysis of global nuclear contamination, where small residual nuclear DNA can be substantial compared to mtDNA at global levels due to difference in the size of two genomes, for the specific site studied using methylation specific primer, as mentioned above, only two potential sites of NUMT exist while copy number of mitochondrial 12S rRNA is in thousands. Although all these data suggests higher possibility of the amplification being obtained from SH-SY5Y mtDNA instead of the nuclear pseudogenes, further confirmatory studies should be conducted by sequencing the methylation specific PCR products and comparing the sequence to mitochondrial genome and potential pseudogene sites and also by following up studies conducted using SH-SY5Y ρ^0 cell line.

A limitation of using HPLC-ESI-MS/MS to determine global mtDNA methylation levels is that the frequency of methylation at specific sites in mtDNA cannot be estimated. However, site-specific studies of mtDNA methylation performed by methylated-DNA-IP and bisulfite-

sequencing have not considered the heteroplasmy that can be present in mtDNA. By studying global mtDNA methylation using HPLC-ESI-MS/MS, we have accounted for the possibility of heteroplasmy present in mtDNA. Further, the method of HPLC-MS/MS entails direct determination of methylated cytosines using the approach of analytical chemistry, making it more sensitive, specific and reproducible compared to other methods which are based on indirect measurements such as bisulphite-sequencing (which requires bisulphite conversion that does not differentiate 5mdC and 5-hydroxy-methyl cytosine), Methylated-DNA-IP and 5-mC-ELISA (requires antibody-mediated- binding to 5mdC in DNA) (Dahl and Guldberg 2003).

In conclusion, we established a direct and specific method to quantify global methylation in mtDNA using HPLC-MS/MS and addressed issues of variable DNA digestion and the possibility of nuclear DNA contamination. Further, we reported for the first time, presence of methylated DNA in gradient-purified SH-SY5Y mitochondria suggesting presence of mtDNA methylation in SH-SY5Y neuroblastoma cells.

2.6 FIGURES

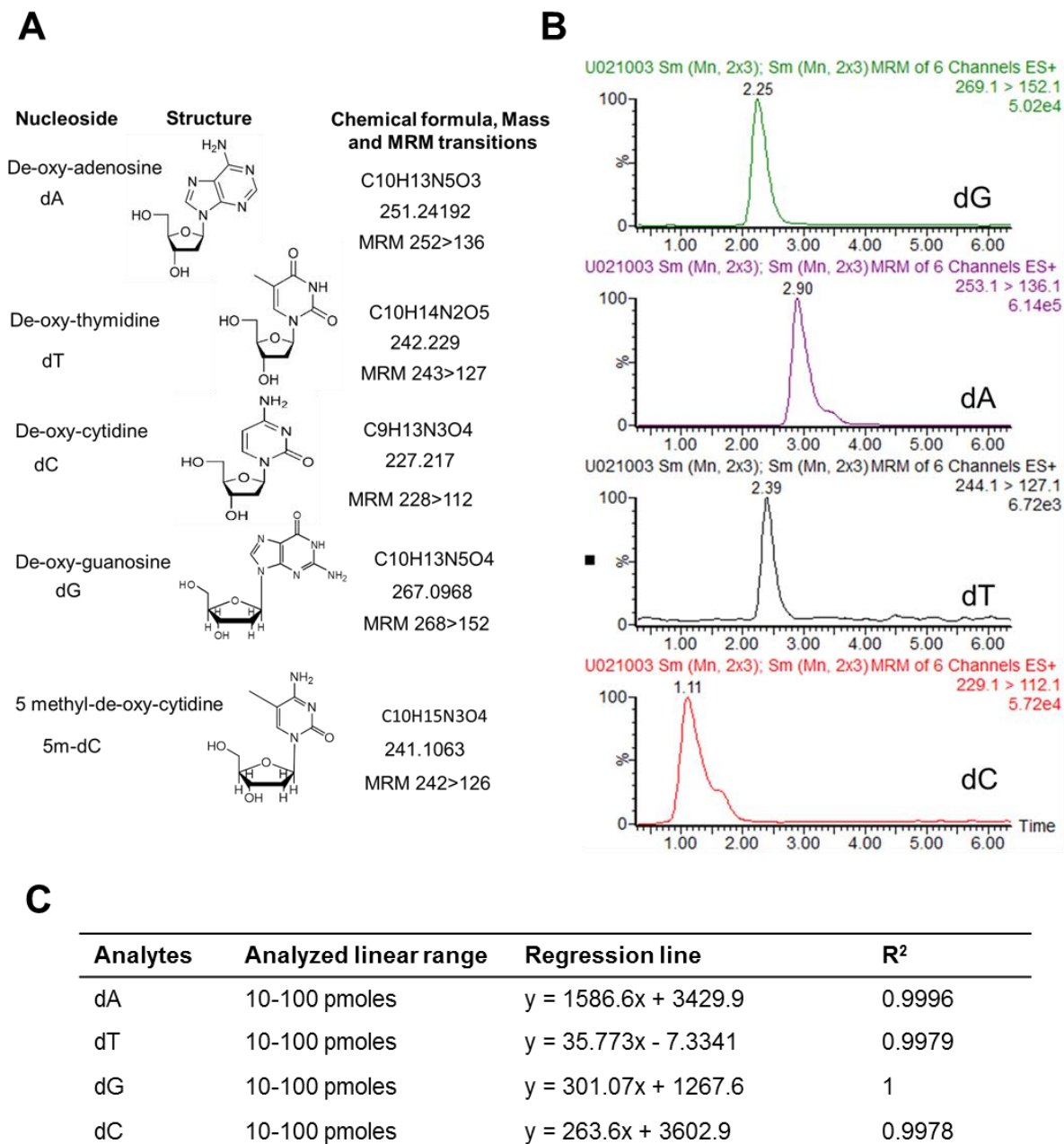


Figure 1: Characterization of dA, dG, dT, dC and 5mdC by HPLC-ESI-MS/MS

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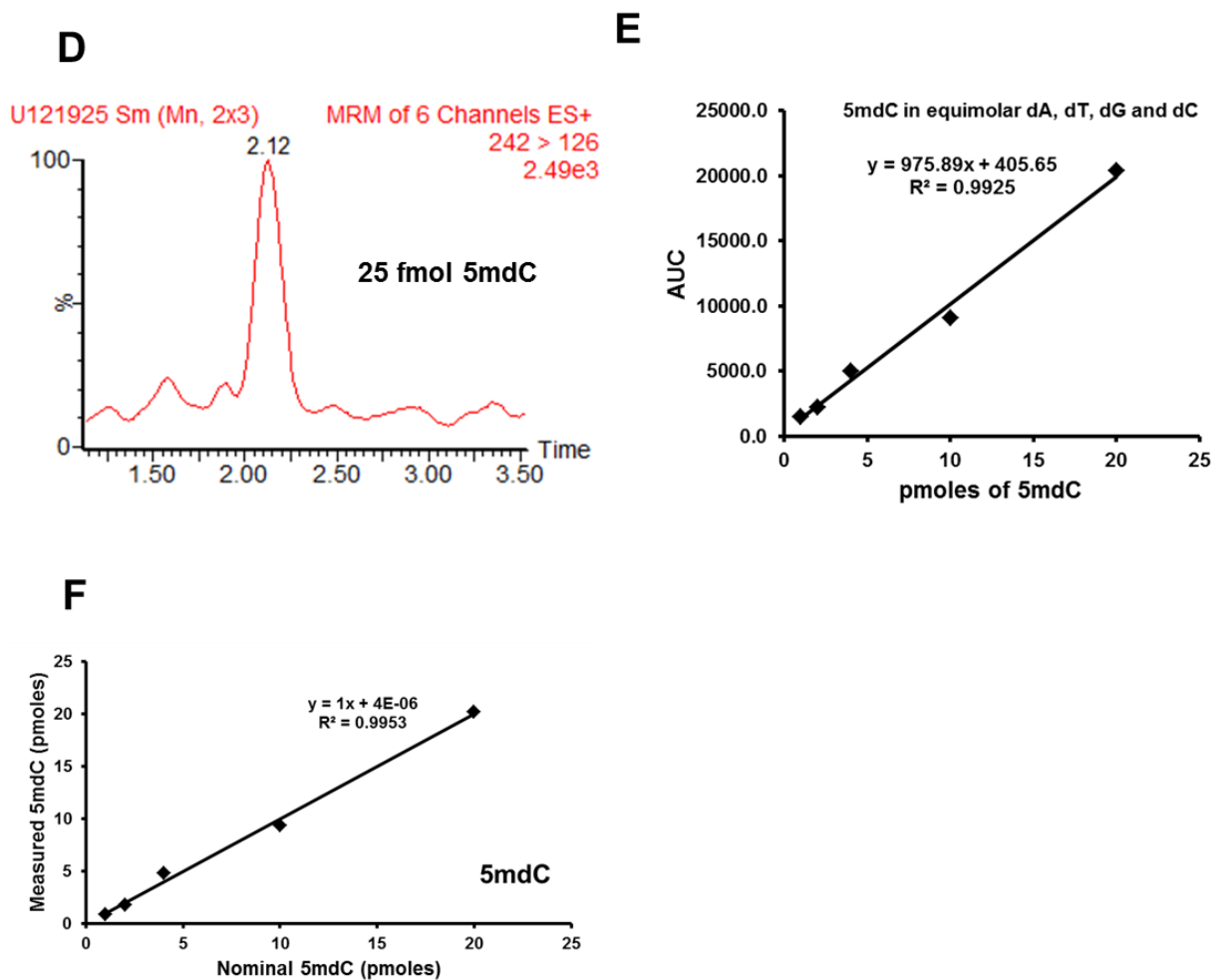
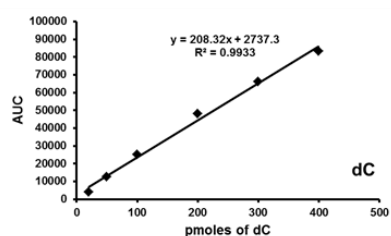
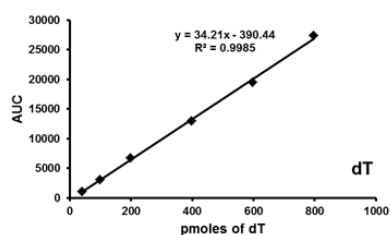
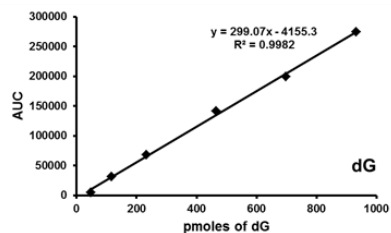
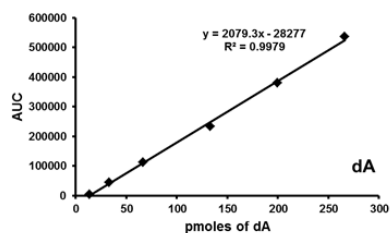


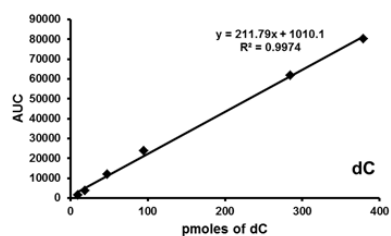
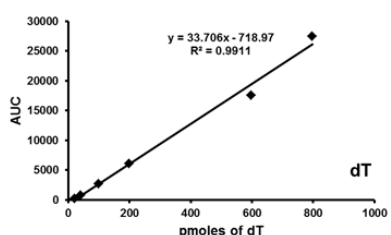
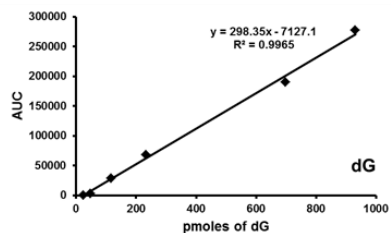
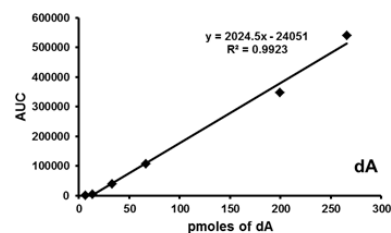
Figure 1 Characterization of dA, dG, dT, dC and 5mdC by HPLC-ESI-MS/MS: (A) Description of nucleosides including structure, molecular weight and the MRM transitions followed for quantification of respective nucleosides. (B) Typical chromatograms obtained from equimolar mixture of dA, dT, dG and dC showing peaks corresponding to MRM transitions followed for dA, dT, dG and dC. (C) Representative linear equations with coefficient of regression derived from calibration curve of each nucleoside which were obtained from

equimolar mixture of dA, dT, dG and dC (10 -100 pmoles). (D) Chromatogram obtained from 25 fmol of 5mdC (LOD) in equimolar mixture of dA, dT, dG and dC showing peak corresponding to MRM transition followed for 5mdC quantification (E) Representative calibration curve for 5mdC (1-20 pmoles) in equimolar mixture of dA, dT, dG and dC (40 pmoles of each). (F) Measured 5mdC levels derived from calibration curve were plotted against nominal 5mdC levels showing proportionality of quantification.

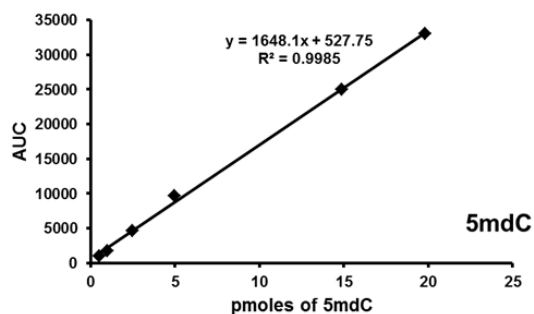
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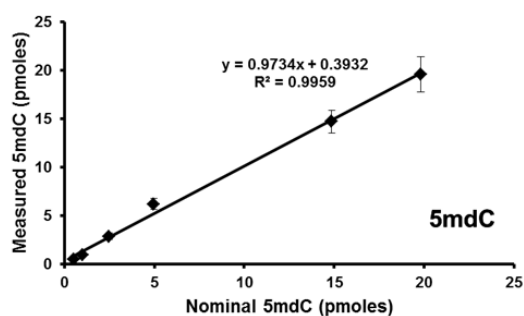
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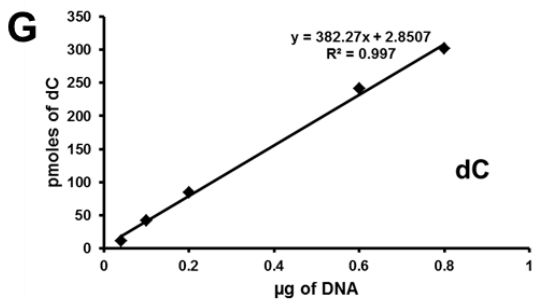
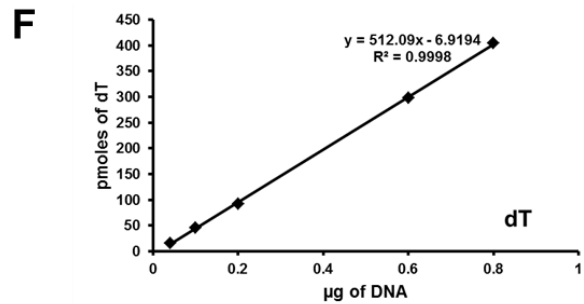
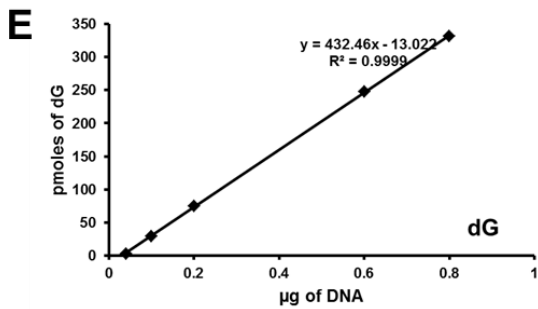
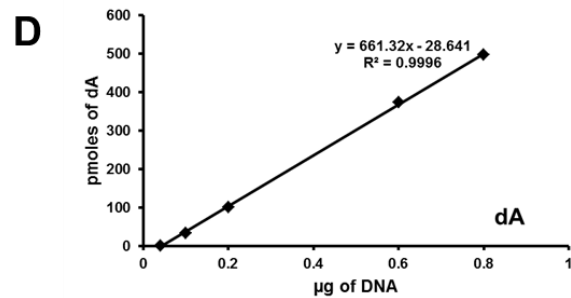
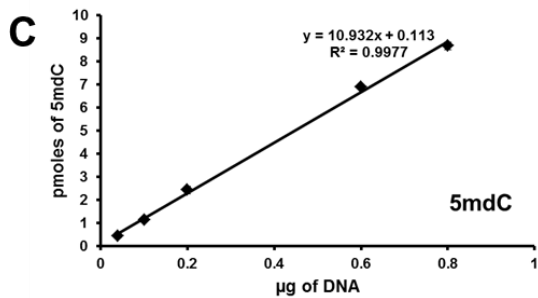
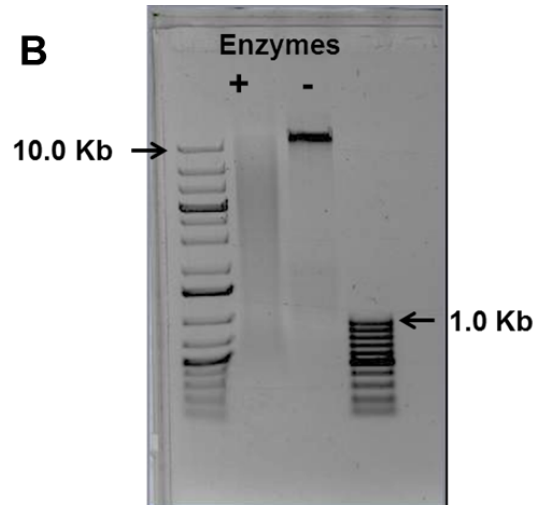
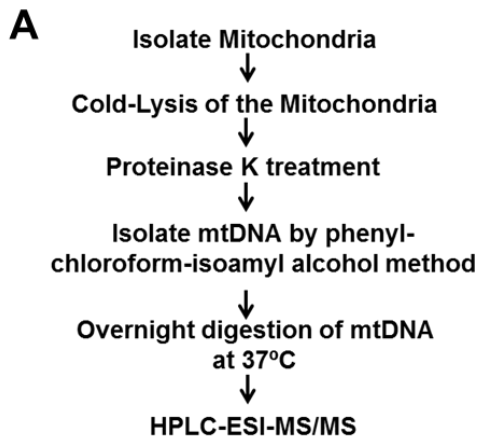


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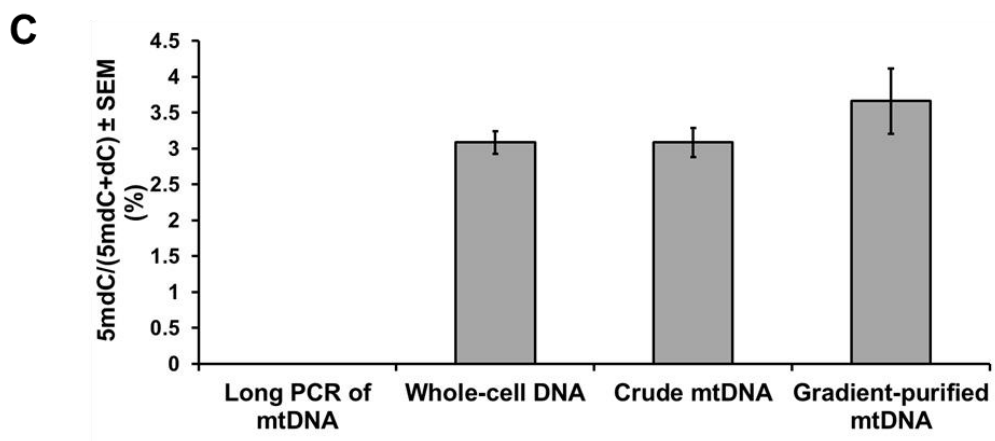
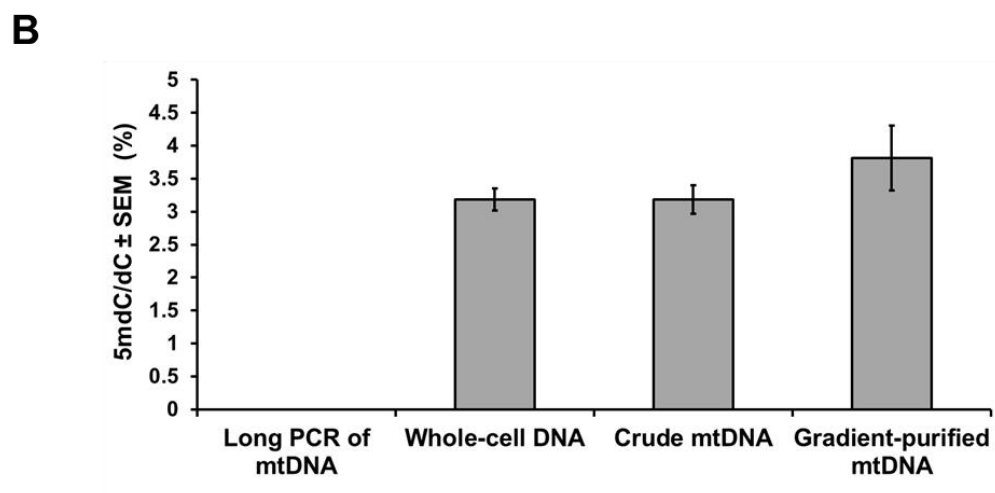
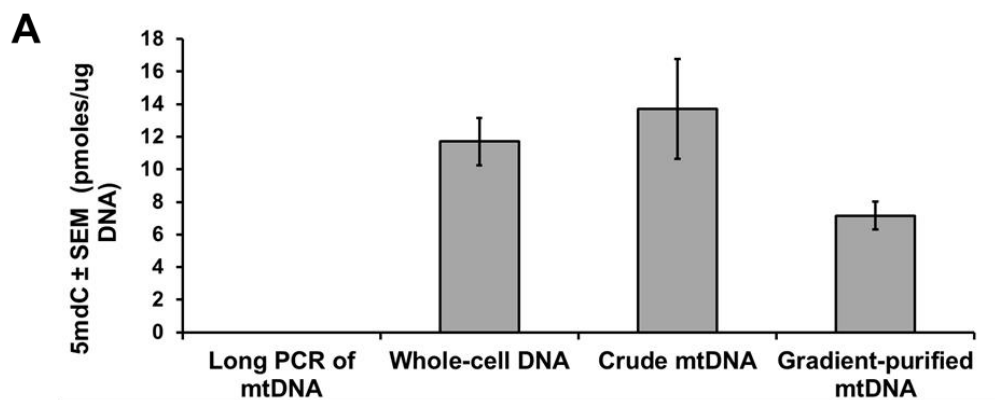
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Figure 2 Linear digestion and quantification of nucleosides in oligo-deoxy-nucleotides containing methylated and un-methylated cytosine: Representative calibration curves plotting nominal amounts against Area under curve (AUC) for dA, dT, dG, and dC derived from digestion products of custom-made (A) un-methylated oligo-deoxy-nucleotide and (B) methylated oligo-deoxy-nucleotide. (C) Representative calibration curve for 5mdC derived from digestion products of custom-made methylated oligo-deoxy-nucleotide showing linear digestion. Total DNA content for analyzed oligo-deoxy-nucleotide ranged from 0.04 to 0.8 μ g and corresponding nominal levels of each nucleoside are shown on X-axis. (D) Measured 5mdC levels (derived from calibration curve) plotted vs. nominal 5mdC levels present in oligo-deoxy-nucleotide before digestion showing proportionality of digestion and quantification.



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Figure 3 Linear and proportional detection of nucleosides in SH-SY5Y mtDNA: (A) Schematic representation of processing samples upstream of HPLC-MS/MS analysis including mtDNA isolation and digestion. (B) Representative image of 1% agarose gel showing the presence of single band for isolated mtDNA before digestion and the resulting smear after enzymatic digestion. (C) – (G) Measured levels of nucleosides (derived from methylated oligo-deoxy-nucleotide calibration curves) plotted against the amount of DNA present in SH-SY5Y mtDNA samples before digestion showing linearity of digestion for each nucleoside. Total DNA content for analyzed SH-SY5Y mtDNA ranged from 0.04 to 0.8 µg.



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Figure 4 Detection of 5mdC in mtDNA extracted from crude and gradient-purified mitochondria: 5mdC was quantitated using HPLC-ESI-MS/MS in ‘Long PCR of mtDNA’, whole-cell DNA and mtDNA extracted from crude and gradient-purified mitochondria, after they were digested into nucleosides. The 5mdC levels are represented as **(A)** absolute values (pmoles/μg DNA) and as percentages of **(B)** 5mdC/dC and **(C)** 5mdC/(dC + 5mdC). At level of injection, 0.4 μg of DNA was used for analysis from each sample.

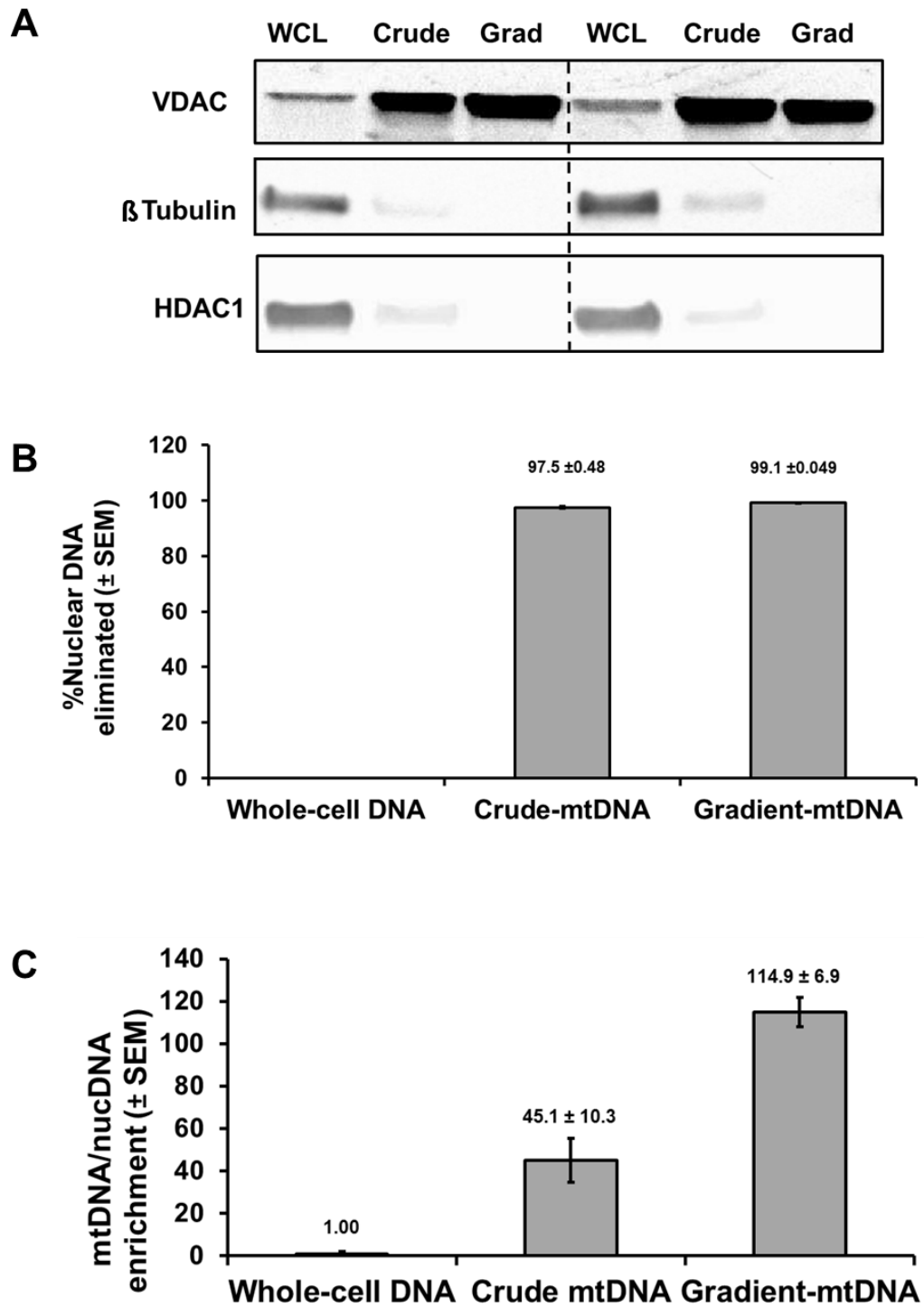


Figure 5: Purity of the preparation of mitochondria and mtDNA extracted from crude and gradient-purified mitochondria

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Figure 5 Purity of the preparation of mitochondria and mtDNA extracted from crude and gradient-purified mitochondria: (A) Whole-cell lysates, mitochondrial lysates of crude and gradient-purified mitochondria of SH-SY5Y were checked for the presence of VDAC, β -Tubulin and HDAC1 to determine purity of the isolated mitochondria using western blot analysis. Whole-cell DNA and mtDNA from crude and gradient-purified mitochondria were extracted from equal number of SH-SY5Y cells. (B) Percentage of nuclear DNA eliminated in mtDNA extracted from crude and gradient-purified mitochondria were determined with respect to whole-cell DNA. (C) Fold-enrichment of mtDNA was determined in mtDNA extracted from crude and gradient-purified mitochondria with respect to whole-cell DNA.

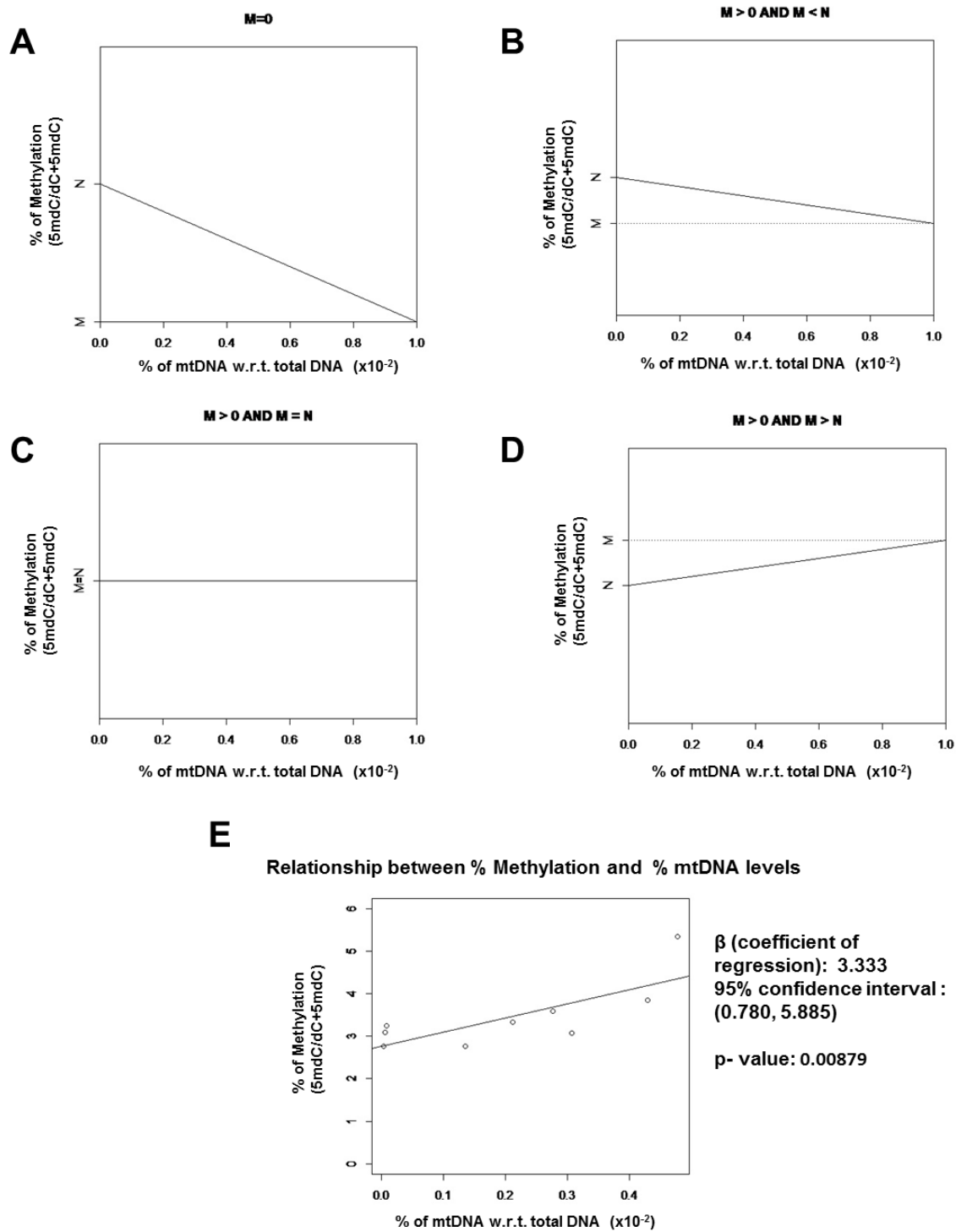


Figure 6 Linear regression analysis of percentage methylation levels and percentage

mtDNA in mitochondrial preparations: M and N are the arbitrary level of methylations for mitochondrial and nuclear DNA respectively. **(A) to (D)** shows scenarios representing relationship between percentage methylation levels and percentage mtDNA based on linear regression modeling when **(A)** $M=0$, **(B)** $M > 0$ and $M < N$, **(C)** $M > 0$ and $M=N$ and **(D)** $M>0$ and $M>N$. **(E)** Linear regression analysis based on experimental data set showing correlation between percentage methylation and percentage mtDNA using preparation of mitochondria with increasing purity.

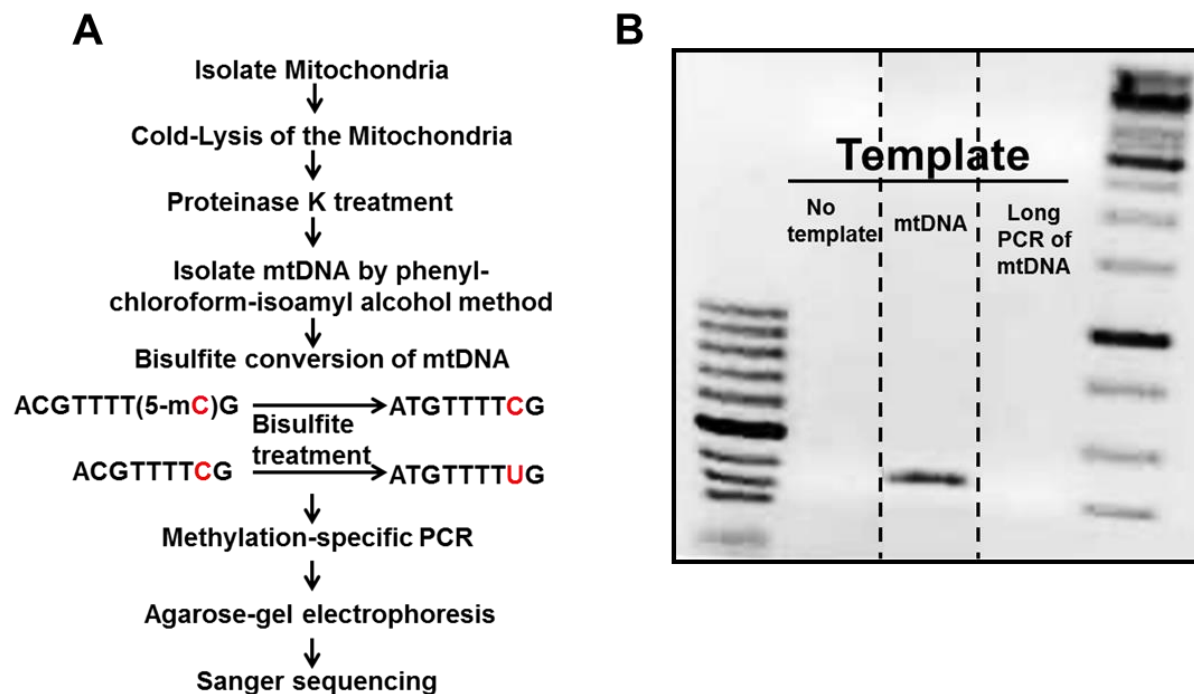


Figure 7 Detection of methylation in mtDNA at 12S rRNA by methylation-specific PCR following bisulfite treatment: (A) Flowchart depicting the isolation of mtDNA, its bisulfite-conversion and amplification using methylation-specific primers and semi-quantitative assessment by agarose-gel electrophoresis. (B) Methylation-specific primers for specific site in 12s rRNA were used to amplify from bisulfite-treated mtDNA and ‘Long- PCR’; subsequent PCR products were electrophoresed through 1% agarose-gel containing ethidium bromide and visualized by UV as shown.

**CHAPTER III: ALTERATIONS IN MTDNA METHYLATION BY DNMT1
OVEREXPRESSION & 5-AZA-DC TREATMENT AND ITS ASSOCIATION WITH
MITOCHONDRIAL TRANSCRIPTION AND MITOCHONDRIAL FUNCTION IN SH-
SY5Y NEUROBLASTOMA CELL LINE**

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Swerdlow

3.1 ABSTRACT

Functional significance of mitochondrial DNA (mtDNA) methylation has largely remained uncharted in the past. However, recent discoveries regarding presence of DNA Methyl-Transferases (DNMTs) in mitochondria, existence of mitochondrial isoform of DNMT1 and studies demonstrating the association of mtDNA methylation with various patho-physiological conditions have led to speculations about contribution of mtDNA methylation in regulating transcription of mtDNA-encoded genes and mitochondrial function, which remain untested. Further, any direct role of DNMTs in altering mtDNA methylation is yet to be established. In this study, we investigated if mtDNA methylation can be altered by different experimental approaches and whether this alteration in mtDNA methylation has any influence on mitochondrial transcription and mitochondrial function in SH-SY5Y neuroblastoma cell line. In order to investigate whether mtDNA methylation can be altered, DNMT1 overexpression and treatment with DNA methylation inhibitor (5-az-2'-deoxycytidine or 5-aza-dC) were implemented as experimental strategies. Global mtDNA methylation was assessed by HPLC-ESI-MS/MS and site-specific methylation of 12S rRNA was examined by methylation-specific primer. DNMT1 was found to be present in purified mitochondria extracted from SH-SY5Y cells. Interestingly, transient overexpression of DNMT1 in SH-SY5Y increased mtDNA methylation at global levels as well at a specific site in 12S rRNA. This corresponded with decreased transcription of mtDNA-encoded genes and reduced basal-mitochondrial oxygen consumption rate upon overexpression of DNMT1. In contrast, treatment of SH-SY5Y by DNA methylation inhibitor (5-aza-dC) reduced levels of mtDNA methylation, which corresponded with increased transcription of mtDNA-encoded genes. However, mitochondrial oxygen consumption rate was reduced after 5-aza-dC treatment, similar to DNMT1 overexpression. In

conclusion, we report that mtDNA methylation is an alterable phenomenon, which can be influenced by levels of DNMT1 and treatment with DNA methylation inhibitor and is associated with changes in mtDNA transcription and mitochondrial function.

3.2 INTRODUCTION

DNA methylation is known to play a key role in regulation of nuclear gene-expression (Bestor 2000, Jones 2012). DNA methylation is catalyzed by a group of enzymes called DNA Methyl-Transferases (DNMTs) that transfer a methyl group from S-Adenosine-Methionine (SAM) to the C5 position of cytosine residue to generate 5-methyl-cytosine (5mdC). DNMT family consists of the *de novo* DNA Methyl-Transferases (Okano, Bell et al. 1999), namely DNMT3A and DNMT3B and the maintenance DNA Methyl-Transferase -DNMT1 (Li, Bestor et al. 1992), which copies the methylation onto the nascent un-methylated strand of DNA formed during DNA replication.

Mitochondria are bi-genomically encoded by two genomes - the nuclear and mitochondrial genome (mtDNA). Along with 24 rRNAs/tRNAs that are essential for mitochondrial translation, mtDNA encodes for 13 proteins required for mitochondrial function. Although nuclear DNA methylation and its role in gene expression are well established, mtDNA methylation and its role in regulating the expression of mtDNA-encoded genes were largely overlooked until some recent studies. Latest reports regarding existence of mitochondrial isoform of DNMT1 (mtDNMT1) (Shock, Thakkar et al. 2011) and localization of DNMTs (including DNMT1 (Chestnut, Chang et al. 2011, Shock, Thakkar et al. 2011, Bellizzi, D'Aquila et al. 2013), DNMT3A (Chestnut, Chang et al. 2011, Wong, Gertz et al. 2013) and DNMT3B (Bellizzi, D'Aquila et al. 2013)) in mitochondria, coupled with possible histone-like role of TFAM (Kanki, Nakayama et al. 2004, Shutt, Lodeiro et al. 2010, Lodeiro, Uchida et al. 2012), have revived interest in studying mtDNA methylation and its functional significance. Mitochondrial isoform of DNMT1 has been reported to be encoded from start sites upstream of the conventional open reading frame (ORF)

and its expression is regulated by NRF1 and PGC1- α , which are transcription factors that control expression of nuclear-encoded genes for mitochondrial biogenesis. Further, mtDNMT1 was found to interact with mtDNA at CpG sites (Shock, Thakkar et al. 2011). However, direct role of mtDNMT1 or any other DNMTs in mtDNA methylation is not yet known.

Alterations in mtDNA methylation have been reported in samples obtained from human subjects with various pathological conditions (such as Non-Alcoholic Fatty Liver Disease (NAFLD) (Pirola, Gianotti et al. 2013), Down-Syndrome (Infantino, Castegna et al. 2011), colorectal cancer (Feng, Xiong et al. 2012), insulin-resistance (Zheng, Linarelli et al. 2015) and cardiovascular disease (Baccarelli and Byun 2015)), during developmental-stages of brain (Ghosh, Sengupta et al. 2014) and upon exposure to air-borne heavy-metals in blood (Byun, Panni et al. 2013) and placenta (Janssen, Byun et al. 2015). Abnormalities of 16S rRNA methylation were found in mtDNA obtained from the skeletal muscle and spinal cord in a mice-model for Amyolateral sclerosis (Wong, Gertz et al. 2013). In few of these reports, correlations of mtDNA methylation were observed with the expression of mtDNA-encoded genes (Feng, Xiong et al. 2012, Byun, Panni et al. 2013, Pirola, Gianotti et al. 2013, Wong, Gertz et al. 2013), thus raising the possibility that mtDNA methylation and transcription of mtDNA-encoded genes are connected. However, no studies have tested a direct link yet. Although, mitochondrial dysfunction is known to be present in the some of the above-mentioned pathological conditions, role of mtDNA methylation in altering mitochondrial function has not been explored either.

In the current study, we investigated whether mtDNA methylation can be altered by overexpression of DNMT1 and treatment with DNA-methylation inhibitor (5-aza-deoxy-Cytosine or 5-aza-dC) in SH-SY5Y cells and if changes in mtDNA methylation are linked with

mitochondrial transcription and mitochondrial function. We have found mtDNA methylation to be an alterable phenomenon, which can be affected by DNMT1 levels or 5-aza-dC treatment and is associated with changes in mtDNA transcription and mitochondrial function.

3.3 MATERIALS AND METHODS

Cell Culture and its harvest

SH-SY5Y neuroblastoma cells were cultured in DMEM containing 25 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), 1% Pen-strep and maintained at 37°C, 5% CO₂. Cell lines growing in the culture flasks or petri-dishes were rinsed with 1X Phosphate Buffered Saline (PBS), trypsinized and then harvested using the growth media. The harvested cells were then pelleted at 800g for 5 minutes at 4°C and subsequently washed with 1X PBS at 4°C for subsequent downstream procedure.

Plasmid preparation and overexpression of DNMT1

PcDNA3/Myc-DNMT1 (Plasmid #36939) and the vector-backbone pcDNA3 were purchased from Addgene in the form of their bacterial stock and grown in Lurea Broth containing 1 µg/mL ampicillin for selection as described previously (Wilkins, Marquardt et al. 2012). The respective plasmids were isolated from the cultures using Endo-Free Plasmid Maxi Kit from Qiagen. SH-SY5Y cells were seeded and lipofectamine-mediated-transient- transfection in optimem medium was carried out at 16 hours as described previously (Wilkins, Marquardt et al. 2012) , 48 hours post-seeding, the transfected cells were harvested for various experimental purposes. Details of different parameters of transfection used for various experimental purposes are listed in the following table.

Experiment	Cell culture vessel	Cell seeding density	Amount of plasmid	Amount of lipofectamine
Protein extraction, RNA isolation	6- well plates	7×10^5	5 μ g	5 μ L
mtDNA isolation	10 cm dish	7×10^6	25 μ g	25 μ L
Oxygen Consumption Stress test	XF seahorse culture plate	5×10^5	0.5 μ g	1 μ L

DNA methylation inhibitor treatment

5-aza-2'deoxyctidine (catalogue # A3656) was purchased from Sigma. SH-SY5Y cells were cultured in the growth medium containing 0, 5, 10 and 15 μ M 5-aza-2'deoxyctidine for 48 hours and maintained at 37°C, 5% CO₂. SH-SY5Y cells were seeded at 15×10^6 in T180 flasks for isolation of mitochondria and subsequently mtDNA was extracted from them. SH-SY5Y cells were seeded at 7×10^5 in 60 mm petri-dishes for extraction of RNA, cDNA synthesis and to determine the transcript levels of mtDNA-encoded genes. SH-SY5Y cells were seeded at 5×10^5 in XF seahorse culture plate for the study of oxygen consumption rate.

Isolation of crude and percoll-gradient purified mitochondria

Crude preparation of mitochondria: Mitochondria were isolated as described previously (Kristian, Hopkins et al. 2006) with some modifications. Briefly, cells growing in a confluent

T180 flask were harvested as described above. The washed cell pellets were then re-suspended in MSHE buffer, collected in a pre-cooled cavitation chamber (nitrogen bomb; Parr Instrument Company, Moline, IL) and subjected to 900 psi for 15 minutes. The cell suspension is collected by releasing the pressure instantaneously and centrifuged at 1000g for 5 minutes at 4°C to pellet the cell debris. The supernatant was collected and spun at high speed of 20000g for 10 minutes at 4°C. The pellet obtained was re-suspended in MSHE and is the crude preparation of mitochondria.

Percoll gradient-purified mitochondria: Gradient-purified mitochondria were isolated in a modified version as described previously (Kristian, Hopkins et al. 2006). Percoll was purchased from GE-Healthcare (catalogue # 28-9038-48) and 100% percoll was prepared by mixing 9 parts of Percoll with 1 part of 1.5M NaCl. The crude pellet of mitochondria obtained is re-suspended in 800 µL of 15% percoll and layered on to a gradient of 50% and 21% percoll. The resulting percoll-gradient underwent ultra-centrifugation in a swing-bucket rotor system (SW 28.1 rotor) at 30,700 g for 15 minutes at 4°C. The pure mitochondria separated between 50 and 21% percoll, was collected, diluted with MSHE and then washed at 20,000 g for 15 minutes at 4°C twice. The pellet obtained is the percoll-gradient purified mitochondria.

Mitochondrial lysate of crude and gradient-purified mitochondria were prepared using M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Protein concentrations for the lysates were determined using a BCA Protein Assay kit (BioRad).

DNA extraction

DNA extraction was done by the method based on organic-solvent extraction of DNA as described previously (Guo, Jiang et al. 2009). Briefly, the sample in ice-cold lysis buffer underwent dounce homogenization, followed by Proteinase K digestion at 55°C for 3h in a water bath. The mixture was spun to remove the non-soluble fractions and the DNA in the supernatant was extracted by standard Phenol-Chloroform-Isoamyl alcohol method. DNA was precipitated using Sodium Acetate and isopropanol at -20°C. The DNA pellet was washed twice with 70% ethanol. Finally, the extracted DNA pellet is then air-dried, re-suspended in nuclease-free water and then quantitated using Infinite M200 (Tecan, San Jose, USA).

SH-SY5Y mtDNA extraction: The mitochondrial DNA was extracted from both the crude mitochondria for determining the amount of mtDNA methylation. Twenty million cells in a T180 flask were seeded and harvested 48 hours later for extraction of mitochondria as described above. The isolated mitochondria were suspended in ice-cold Lysis buffer for DNA extraction as described above.

Digestion of DNA

Oligo-deoxy-nucleotides with known amount of dC and 5mdC and mtDNA obtained from SH-SY5Y cells that were treated with 5-az-dC and in which DNMT1 was overexpressed, were digested as described previously (Quinlivan and Gregory 2008). Briefly, known amount of the DNA of interest (2 µg) was digested in 100 µL reaction volume, using the enzymes- 2.5 U Benzonase (Sigma E1014), 3 mU Phosphodiesterase I (Sigma P3243) and 2U Alkaline Phosphatase (Sigma P7923) in Tris-HCl (20 mM. pH 7.9) containing 1 mM NaCl and 0.2 mM MgCl₂ at 37°C for 20-24 hours.

HPLC-ESI-MS/MS

Reagents: 5-Methyl-2'-deoxycytidine (5MdC) was purchased from Berry & Associates, Dexter, MI (Catalogue# PY7635). The four other deoxyribonucleosides- 2'-deoxycytosine (dC), 2'-deoxyguanosine (dG), 2'-deoxyadenosine (dA), and 2'-deoxythymidine (dT), were purchased from Sigma Chemical Co. (St. Louis, MO).

Apparatus: Waters Acquity "Classic" (Waters Corp. Milford, MA) UPLC system was used with an Altima C18 column that had internal diameter of 2.1 mm and length of 50 mm, with sorbent particles of size 3.5 μm (Grace, Columbia, MD). Triple quadrupole mass-spectrometer (Quattro Ultima Micromass Ltd. Manchester, UK) was coupled with the UPLC system through electron-spray probe for detection and quantification of nucleosides.

LC-ESI-MS/MS Procedure: Released nucleosides in the digestion buffer of a particular sample of interest were separated by UPLC for MS/MS detection, adapting a general strategy used for separating a complex mixture of nucleosides. The injection volume used was 20 μL . LC separation was performed at a flow rate of 300 $\mu\text{L}/\text{minute}$. Two buffers, A: 10 mM ammonium acetate (NH_4OAc) and B: 10% Methanol comprised the mobile phase that was used for elution of the nucleosides using the following gradient:

Time (min)	A%	B%
0.0	99	1
1	99	1
2	90	10
9	20	80
11	99	1

The mobile phase effluent were passed through the electrospray probe (source block temperature: 110°C, desolvation temperature: 200°C and cone voltage: 50V) and tandem mass spectra was acquired on triple quadrupole in multiple reaction monitoring (MRM) mode. The first quadrupole (*Q1*) that acts as a mass-filter, allowed a parent ion of a specific mass-to-charge ratio to enter the second quadrupole. The second quadrupole (*Q2*) is the collision cell, where Argon gas at a pressure of 2e-3 mbar was used to fragment the ions through the process of collision-activated disassociation (collision energy: 15 eV). The resulting fragment ions were transmitted to the third quadrupole (*Q3*), where only the daughter ions of a desired mass-to-charge ratio were allowed to pass and reach the detector for further quantification to compose the tandem mass spectra. Selected ion recordings for 5-Methyl-2'-deoxycytidine methylated (5MdC) was 242.1 (parent ion) >126.1 (daughter ion) and for the other nucleosides were dC: 229.1>112.1, dT: 244.1>127.1, dA: 253.1>136.1, dG: 269.1>152.1 with 0.1 sec dwell time. To monitor instrument performance, every batch of samples were punctuated with duplicate preparations of 5MdC standard curve, ranging from 1 to 20 pmoles in a mixture of 40 pmoles of each of the nucleosides dA, dT, dG and dC in 10 mM NH₄OAc as well as only 10mM NH₄OAc as the blank. Further, custom-made oligo-deoxy-nucleotide containing known amount of dC and 5mdC were digested as described above and digestion products was used for generating standard curve to calculate the amount of 5mdC in test samples.

Long PCR of human mtDNA

SH-SY5Y mtDNA was extracted and used as template to generate a PCR product of 15.745Kb long to represent the un-methylated version of human mtDNA as described previously (Binder, Dunn et al. 2005) using Expand Long Range, dNTPack (Roche Diagnostics) that served as a

negative control for methylation in methylation-specific primer assay. The PCR product thus obtained was purified using Qiaquick PCR purification kit (Qiagen) before using it for HPLC-ESI-MS/MS assay, following digestion and for methylation-specific PCR assay, following bisulfite-conversion.

Bisulfite conversion and Methylation-specific PCR assay

Bisulfite conversion: The extracted mtDNA of interest and ‘Long PCR’ was treated with bisulfite using EZ DNA methylation-Gold kit (Zymoresearch, Irvine, CA, USA). Bisulfite sulphonates and then deaminates the un-methylated Cytosine to produce uracil in DNA. Thus methylated cytosines are protected from the conversion to uracil, allowing the use of site-specific PCR amplification i.e. methylation-specific PCR. Briefly, 350 ng of mtDNA in 20 µL nuclease-free water was mixed with 130 µL of CT Conversion reagent and was placed in the thermal cycler where the program consisted of initial denaturation at 98°C for 10 minutes, followed by conversion at 64°C for 2.5 hours. The converted solution of mtDNA was mixed with binding buffer to allow its binding to the column. A high speed spin was followed by washes, desulphonation for 20 minutes at room temperature and then finally eluted using the Elution buffer to obtain the bisulfite-treated DNA.

After obtaining the converted mtDNA, the following primers were used to amplify a region of 12S rRNA using the following PCR program. The primers were designed using the bisulfite treated version of the Cambridge reference sequence of human mtDNA (Anderson, Bankier et al. 1981).

F: AGTTATAAGTTTAAAATTTAAAGGATTTGGC

R: ACTTAAAAAAAAATAACGAACGATATATACGCG

PCR set up: .4 µM of the forward and reverse primer was used to amplify to obtain a 320 bp amplicon using ZymoTaq Premix (Zymoresearch, Irvine, CA, USA).

PCR program: Initial denaturation of 10 mins at 95°C was followed by 30 cycles of denaturation at 95 °C for 30 secs, annealing at 51.5°C for 45 secs and synthesis at 72°C at 45 secs, with a final elongation step of 7 mins at 72°C.

Agarose-gel-electrophoresis: The subsequent PCR products obtained were electrophoresed through 1% agarose gel containing 0.5 µg/ml ethidium-bromide and visualized under ultraviolet light using a Bio-Rad ChemiDoc Imager.

RNA extraction

RNA extraction was performed using TRI Reagent solution (Life technologies AM9738). Briefly, supernatant was removed and TRI was added to harvest the cells. The homogenate was incubated at room temperature to allow dis-association of the nucleoprotein complexes. Chloroform was used to separate the protein from the nucleic acids. RNA was precipitated using isopropanol; the pellet obtained was washed with 70% ethanol and then air-dried before re-suspending in nuclease-free water. The extracted RNA is then further quantitated using Infinite M200 (Tecan, San Jose, USA) and ran on gel to assess its quality.

cDNA synthesis

1 µg of RNA was used to synthesize cDNA using iScript Reverse transcription 5X super-mix in a 20 µL reaction as per the recommendations in the protocol (Biorad 170-8840). The 20 µL

reaction was then diluted 5 times by adding 80 μ L nuclease-free water. 2 μ L of the diluted cDNA solution was used for every qPCR reaction.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and ready-to-use TaqMan Gene Expression Assays (Applied Biosystems). We quantified mRNA levels deriving from the following genes: DNMT1, MT-12S rRNA, MT-16S rRNA, MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND5, MT-ND6, MT-COX1, MT-COX2, MT-CytB and MT-ATP6. GAPDH or β -Actin was used as an internal loading control.

Quantitative real-time PCR (qPCR) was performed using the Sybr Green Universal PCR Master Mix (Applied Biosystems, Foster City, CA) for quantifying mRNA levels of MT-DNMT1 using primers as used in (Dzitoyeva, Chen et al. 2012) and HPRT was used as an internal loading control.

qPCR amplification was determined utilizing an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems).

Protein lysates from mitochondrial and cytosolic fractions

Upon overexpression of DNMT1, protein lysates of mitochondrial and cytosolic fractions were prepared as described previously (Wilkins, Marquardt et al. 2012) using the Mitochondrial/Cytosol Fractionation kit from Biovision. Briefly, cells were harvested and re-suspended in 1X Cytosolic extraction buffer, incubated on ice for 10 minutes and homogenized by dounce-homogenization. The homogenate was spun at 700 g for 10 minutes at 4°C and the

pellet obtained is the nuclear compartment. The supernatant was collected and spun again at 10000g for 30 minutes at 4°C. The pellet obtained is the mitochondria whereas the supernatant is the cytosolic fraction. Protein concentrations for all the lysates were determined using a BCA Protein Assay kit (BioRad).

Immunoblotting

Whole cell lysates were prepared by M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Protein concentrations for all the lysates were determined using a BCA Protein Assay kit (BioRad).

Equal amount of proteins from the protein lysates were resolved by electrophoresis in pre-cast 4–12% gels (BioRad), and then transferred to polyvinyl di-fluoride (PVDF) membranes. Non-specific binding was blocked by gently agitating the membranes in 5% non-fat milk and 0.1% Tween in PBS (PBST) for 1 hour at room temperature. Blots were subsequently incubated in 5% bovine serum albumin (BSA) containing 0.1% Tween with the designated primary antibody overnight at 4°C with gentle agitation. After PBST washes, the blot was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature with gentle agitation. After PBST washes, the blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Chemiluminescence signals were detected using a Bio-Rad ChemiDoc Imager.

Primary antibodies for DNMT1, VDAC, β -Tubulin, GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA) and that of HDAC1 was purchased from Pierce (Thermo Scientific, Eugene, OR, USA). All of the antibodies were used at a 1:1000 dilution.

Oxygen consumption rate (OCR) studies

Approximately 50,000 cells of SH-SY5Y cells were seeded in the wells of Seahorse XF cell culture microplates (Seahorse Bioscience, Billerica, MA) and standard two-step seeding procedure was performed as per the manufacturer's recommendations. The XF cell culture microplates were placed in a 37°, 5% CO₂ incubator for the cells to proliferate. The assays were done 48 hours post seeding. Twenty four hours post seeding, the cells were starved off FBS into a buffered DMEM medium containing 5mM glucose. On the assay day (48 hours post seeding), before placing culture microplates in the Seahorse Analyzer, the cells were washed with unbuffered DMEM containing 5mM glucose and 2 mM pyruvate and then placed in the same medium for an hour in an incubator without CO₂ at 37°C for degassing. OCR respiration measurements were collected after every cycle of 3 minutes of mixing, 2 minutes of wait time, followed by measurements for 3 minutes by the Seahorse Analyzer and the entire assay was completed in twelve such cycles as depicted in the following table.

Injection #	Cycles	Description
	1 st – 3 rd cycles	Total OCR was measured and 3 rd cycle reading was used for analysis.
1	3 rd - 6 th cycles	1 µM Oligomycin was injected after the 3 rd cycle. The post-oligomycin OCR was measured and 6 th cycle reading was used for analysis..
2	7 th – 9 th cycles	0.3 µM FCCP was injected after the 6 th cycle. The post-FCCP OCR was measured and 9 th cycle reading was used for analysis.
3	9 th -12 th	Mixture of 0.1 µM Rotenone and 0.2 µM Antimycin A was injected after

	cycle	the 9 th cycle. Non-mitochondrial OCR was measured and 12 th cycle reading was used for analysis.
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The non-mitochondrial OCR was subtracted from the total OCR to yield the mitochondrial OCR. The proton-leak OCR was obtained by subtracting the non-mitochondrial OCR from the post-oligomycin OCR whereas the maximal OCR was calculated by subtracting the non-mitochondrial OCR from the post-FCCP OCR. After OCR measurements were completed, the cell mass in each well was estimated by measuring the total protein in each well (DC Protein Assay, BioRad). OCR values from each well were normalized to the amount of protein in that well to yield a final corrected OCR in pMol O₂/minute/μg protein.

Data analysis

Data are expressed as means ± standard error of the means (SEM). To compare means between two groups we used two-way, unpaired Student's t-tests, with p-values of <0.05 considered significant.

3.4 RESULTS

Presence of DNMT1 in SH-SY5Y mitochondria and its overexpression

DNMT1 is one of the members of the family of DNA methyl transferases that transfer methyl group to the cytosine in DNA to yield 5-methyl-cytosine (5mdC). Recently, DNMT1 localization in mitochondria was reported for several cell lines (Chestnut, Chang et al. 2011, Shock, Thakkar et al. 2011, Bellizzi, D'Aquila et al. 2013). Additionally, existence of mitochondrial isoform of DNMT1 (mtDNMT1) was also reported recently, which was found to bind mtDNA at CpG sites

(Shock, Thakkar et al. 2011). However, role of DNMT1 in mtDNA methylation is not yet established which was investigated in the current study. To begin with, we isolated crude fractions of mitochondria from SH-SY5Y cells and found DNMT1 to be present in them. In order to rule out the possibility of contamination of nuclear DNMT1 in our crude fractions of mitochondria, we extracted gradient-purified mitochondria by a percoll-gradient. DNMT1 was also found to be present in gradient-purified mitochondria isolated from SH-SY5Y cells (**Figure 1A**). Further, the purity of the preparation of each mitochondrial fraction (crude and gradient-purified mitochondria) was determined by the presence of VDAC (mitochondrial-marker) and HDAC1 (nuclear-marker). These results indicate the presence of DNMT1 in the mitochondria of SH-SY5Y cells.

Next, we transiently overexpressed DNMT1 in SH-SY5Y cells in order to study its effects on mtDNA methylation and subsequently mitochondrial transcription and mitochondrial function. Relative mRNA levels of DNMT1 (**Figure 1B**) as well as mtDNMT1 (**Figure 1C**) were significantly elevated in cells transfected with the plasmid DNA containing DNMT1 gene in comparison to the cells transfected with the empty vector- backbone. DNMT1 protein was found to be increased in the cytosolic, mitochondrial, nuclear fractions as well as whole-cell lysates of the cells transfected with the plasmid DNA containing DNMT1 gene in comparison to the cells transfected with the empty vector-backbone as depicted in **Figure 1D**. The purity of the preparation of each fraction was determined by the presence of VDAC (mitochondrial-marker), HDAC1 (nuclear-marker) and GAPDH (cytosolic-marker). Cells transfected with the empty vector backbone will be referred to as “control” whereas the cells that were transfected with the vector backbone containing DNMT1 gene will be referred to as “oDNMT1”.

Overexpression of DNMT1 increases mtDNA methylation

Next, we investigated whether mtDNA methylation was affected by overexpression of DNMT1 in SH-SY5Y cells. Using HPLC-ESI-MS/MS, total mtDNA methylation was measured in ‘control’ and ‘oDNMT1’ cells. Absolute 5mdC levels were found to be significantly increased (about 2.5 fold) in mtDNA from ‘oDNMT1 cells’ compared to ‘control cells’ as shown in **Figure 2A**. Absolute 5mdC levels were further normalized by the amount of dC and the sum of dC and 5mdC. Both the percentages were significantly higher in the mtDNA from ‘oDNMT1 cells’ than ‘control cells’ as shown in **Figure 2B-C**. We also examined methylation at a specific site in 12S rRNA in bisulfite-treated mtDNA from both the ‘oDNMT1’ and ‘control’ cells using methylation specific primers. A single band of PCR product with remarkably higher density was amplified from bisulfite-treated mtDNA of ‘oDNMT1 cells’ compared to ‘control cells’ as shown in **Figure 2D**. No band was detected in the lane that was loaded with PCR product obtained by using the un-methylated “Long PCR” as a template (negative-control) with the methylation specific primers. These results indicate that overexpression of DNMT1 in SH-SY5Y cell line increases mtDNA methylation. Also, this suggests that mtDNA methylation is an ongoing process in the mitochondria that can be further stimulated by overexpression of DNMT1.

Overexpression of DNMT1 decreases mtDNA transcription

MtDNA encodes for 2 ribosomal RNAs, 22 tRNAs and 13 mRNAs which contribute to the formation of the apparatus essential for oxidative phosphorylation, a prime function of mitochondria (Scarpulla 2008). We examined if the increased methylation of mtDNA by DNMT1 overexpression had any correlation with transcription levels of mtDNA-encoded genes.

Relative expression levels of mitochondrial ribosomal genes -12S rRNA and 16S rRNA were examined and found to be significantly decreased in ‘oDNMT1 cells’ as shown in **Figure 3A and 3B**, respectively. Relative mRNA levels of several Complex I encoding genes such as MT-ND1, MT-ND2, MT-ND3, MT-ND5 and MT-ND6 were also significantly reduced in ‘oDNMT1 cells’ compared to ‘control cells’ as represented in **Figure 3C-E, G-H**, however that of MT-ND4 (**Figure 3F**) were not significantly different between the two groups. Further, relative mRNA levels of MT-CytB, which is a single gene present in mtDNA that codes for Complex III, was significantly reduced in ‘oDNMT1 cells’ than ‘control cells’ as shown in **Figure 3I**. Similarly, relative mRNA levels of MT-COX2 (**Figure 3K**) and MT-ATP6 (**Figure 3M**) that encode for Complex IV and ATP synthase, were assayed and found to be significantly reduced as well in ‘oDNMT1 cells’ compared to ‘control cells’. However, relative mRNA levels of MT-COX1 (**Figure 3J**) and MT-COX3 (**Figure 3L**) encoding for Complex IV were found to be comparable between ‘control cells’ and ‘oDNMT1 cells’. These results indicate that overexpression of DNMT1 in SH-SY5Y cells reduces the levels of mtDNA transcription in general; suggesting that increase in mtDNA methylation upon overexpression of DNMT1 is associated with decreased mitochondrial transcription.

Overexpression of DNMT1 affects mitochondrial oxygen consumption rate (OCR)

As proteins that form the mitochondria are encoded by both nuclear as well as mitochondrial genes, alteration in mitochondrial transcription can affect mitochondrial function. Since, we observed an overall decrease in transcription of mtDNA-encoded genes after overexpression of DNMT1; we investigated if mitochondrial function is also affected. We used Seahorse XF Analyzer to determine oxygen consumption rates (OCR) (a measure of mitochondrial function)

in the 'control' and 'oDNMT1' cells in unstressed assay conditions. At the end of the assay, rotenone (a Complex I inhibitor) and Antimycin (a Complex III inhibitor) was injected that blocked the mitochondrial respiration, and thus provide the non-mitochondrial OCR. The basal mitochondrial OCR was obtained by subtracting the non-mitochondrial OCR from the total OCR. Under unstressed assay conditions, 'oDNMT1 cells' had significantly lower total OCR than the 'control cells' (34% decrease) (**Figure 4A**). Further, the basal-mitochondrial OCR was significantly decreased in the 'oDNMT1 cells' when compared to the 'control cells' (36% decrease) (**Figure 4B**). However, the non-mitochondrial OCR was similar between the 'oDNMT1 cells' and 'control cells' (**Figure 4C**). These results indicate that overexpression of DNMT1 reduces basal-mitochondrial oxygen consumption rate, suggesting that increase in mtDNA methylation is associated with mtDNA transcription and might be responsible for affecting mitochondrial oxygen consumption.

DNA methylation inhibitor decreases mtDNA methylation

Decitabine or 5-Aza-2'-deoxycytidine (5-aza-dC) is a modified cytosine that cannot be methylated at C5 position (site where cytosine is methylated), gets incorporated in nascent DNA during replication and thus, leads to demethylation or hypo-methylation. 5-aza-dC is commonly used as a tool to inhibit nuclear DNA methylation (Momparler, Cote et al. 1997, Momparler 2005) but whether it can inhibit mtDNA methylation is not known. We examined if 5-aza-dC treatment of SH-SY5Y cells decreased mtDNA methylation. Using HPLC-ESI-MS/MS, total mtDNA methylation was found to be significantly decreased (36% reduction) in mtDNA isolated from SH-SY5Y cells treated with 5-aza-dC (5-15 μ M) compared to the vehicle-treated (0 μ M) cells as shown in **Figure 5A**. Absolute 5mdC levels were further normalized by the amount of

dC and the sum of dC and 5mdC. Both the percentages were significantly reduced (50% reduction) in the mtDNA isolated from 5-15 μ M 5-aza-dC treated cells compared to vehicle-treated cells as shown in **Figure 5B-C**. 5 μ M 5-aza-dC was sufficient to produce maximal decrease in global mtDNA methylation levels such that increase in 5-aza-dC concentration did not decrease methylation levels further. We also examined methylation at a specific site in 12S rRNA in bisulfite-treated mtDNA from 5-aza-dC treated cells using methylation-specific primers. A single band of PCR product with dose dependent decrease in band intensity were obtained from mtDNA of 5-aza-dC treated cells compared to vehicle treated cells as shown in **Figure 5D**. No band was detected in the lane that was loaded with PCR product obtained while using the un-methylated “Long PCR” as a template (negative control) with the methylation-specific primers. These results indicate that 5-aza-dC treatment in SH-SY5Y cell line decreases mtDNA methylation. Also, this suggests that mtDNA methylation is an ongoing process in the mitochondria that can be inhibited by 5-aza-dC treatment.

DNA methylation inhibitor increases mtDNA transcription and affects mitochondrial oxygen consumption rate (OCR)

Next, we examined if the decreased methylation of mtDNA by 5-aza-dC treatment had any correlation with mtDNA transcription and mitochondrial function. Relative expression levels of mitochondrial ribosomal genes - 12S rRNA (**Figure 6A**) and 16S rRNA (**Figure 6B**) were significantly elevated after 5-aza-dC treatment. Further, relative mRNA levels of genes that code for Complex I such as MT-ND1, MT-ND3 and MT-ND4 (**Figure 6C-E**) increased significantly after treatment with 5-aza-dC, but were not significantly different for MT-ND5 and MTND6 (**Figure 6F-G**). Additionally, after 5-aza-dC treatment, relative mRNA levels of all three

mtDNA genes that encode Complex IV (namely, MT-COX1, MT-COX2 and MT-COX3) were found to be significantly elevated (**Figure 6H-J**). Further, mRNA levels of MT-CytB and MT-ATP6 that code for Complex III and ATP synthase, respectively increased significantly after 5-azadC treatment (**Figure 6K-L**). These results indicate that treatment with DNA methylation inhibitor 5-aza-dC in SH-SY5Y cells increases the levels of transcription for mtDNA-encoded genes in general; suggesting that decrease in mtDNA methylation upon 5-aza-dC treatment is associated with increased mitochondrial transcription.

Further, mitochondrial function was investigated using Seahorse XF Analyzer by determining oxygen consumption rates (OCR) upon 5-aza-dC treatment (5-15 μ M). Under unstressed assay conditions, 5-aza-dC treatment significantly decreased total OCR (40% reduction) (**Figure 7A**). Further, the basal-mitochondrial OCR was significantly decreased upon 5-aza-dC treatment when compared to the vehicle-treated cells (42% reduction) (**Figure 7B**). The non-mitochondrial OCR was found to be comparable for 5 and 10 μ M 5-aza-dC treated cells with respect to the vehicle-treated, but was significantly lower in the 15 μ M treated cells (**Figure 7C**). These results indicate that 5-aza-dC treatment reduces mitochondrial oxygen consumption rate, suggesting that decrease in mtDNA methylation by 5-aza-dC treatment is associated with increased mtDNA transcription and might have a role to play in altering mitochondrial function.

3.5 DISCUSSION

Involvement of mtDNA methylation in regulating transcription of mtDNA-encoded genes and subsequently mitochondrial function is being contemplated, ever since DNA Methyl - Transferases were reported to be present in mitochondria (Shock, Thakkar et al. 2011). In this study, we investigated whether mtDNA methylation is alterable by overexpression of DNMT1

and 5-aza-dC treatment in SH-SY5Y cells and if there are any associated changes in mitochondrial transcription and function. MtDNA methylation was found to increase after overexpression of DNMT1 and decrease after 5-aza-dC treatment at both global and site-specific levels. Further, alteration of mtDNA methylation was accompanied with changes in transcription of mtDNA-encoded genes and mitochondrial-oxygen consumption rate.

Previous studies have reported DNMT1 localization in mitochondria of human cell lines such as HCT116 (Shock, Thakkar et al. 2011), HEK293 (Chestnut, Chang et al. 2011), HeLa (Bellizzi, D'Aquila et al. 2013) and mouse cell lines such as MEF (Shock, Thakkar et al. 2011), 3T3-L1 (Bellizzi, D'Aquila et al. 2013) and NSC34 (Chestnut, Chang et al. 2011). Recently, existence of mitochondrial isoform of DNMT1 also known as the mtDNMT1 was reported, which was also found to interact with mtDNA at CpG sites (Shock, Thakkar et al. 2011). However, any direct role of DNMT1 or its mitochondrial isoform in methylation of mtDNA is yet to be determined. To the best of our knowledge, we report for the first time that DNMT1 is present in SH-SY5Y mitochondria and that its overexpression increases methylation of mtDNA at global levels as well as at 12S rRNA. Concomitant increases of DNMT1 levels in mitochondria and mtDNA methylation, suggests that DNMT1 might be responsible for mtDNA methylation. However, this does not refute the possibility of presence of other DNA methyl transferases that could be involved in methylating SH-SY5Y mtDNA. We also report a significant decrease in mtDNA methylation upon treatment with DNA-methylation inhibitor (5-aza-dC) at global levels and at 12S rRNA. Both of these observations put together suggest that mtDNA methylation is an alterable and on-going phenomenon that can be both stimulated as well as inhibited. As HPLC-ESI-MS/MS assay used in our study cannot differentiate nucleosides obtained from mtDNA or nuclear DNA and even though the mtDNA has been extracted from isolated mitochondria,

chances of nuclear DNA contamination cannot be excluded completely. However, we have observed that the mtDNA prepared in this manner is enriched by 50 fold for mtDNA with respect to total DNA and 99.71 % of the nuclear DNA is eliminated. Moreover, changes in mtDNA methylation globally by HPLC-ESI-MS/MS were corroborated by similar changes at specific site in mtDNA as detected by methylation-specific PCR assay, suggesting that confounding presence of nuclear DNA contamination would not have affected the interpretation of our results. Additionally, bisulfite-sequencing of mtDNA following overexpression of DNMT1 as well as 5-aza-dC treatment might provide an in-depth insight on about sites where methylation was altered, which was not pursued in the current study.

Previous studies have only correlated the expression of mtDNA-encoded genes with levels of mtDNA methylation in various pathological conditions such as colorectal cancer (Feng, Xiong et al. 2012) and non-alcoholic fatty liver disease (Pirola, Gianotti et al. 2013), but direct evidence supporting the role of mtDNA methylation in controlling the mitochondrial transcription is lacking. We found for the first time that increasing global mtDNA methylation by overexpressing DNMT1 decreased the transcript levels of mtDNA-encoded genes, whereas decreasing mtDNA methylation upon treatment with DNA-methylation inhibitor increased the transcript levels of mtDNA-encoded genes. Moreover, we found that relative expression levels of 12S rRNA inversely correlated with the levels of mtDNA methylation in 12S rRNA as studied by methylation-specific PCR assay. Our observations about the association of altered transcript levels of MT-ND6 with DNMT1 levels are coincident with other correlative findings (Shock, Thakkar et al. 2011, Pirola, Gianotti et al. 2013). Thus, our results support the hypothesis that mtDNA methylation can regulate the expression of mtDNA-encoded genes.

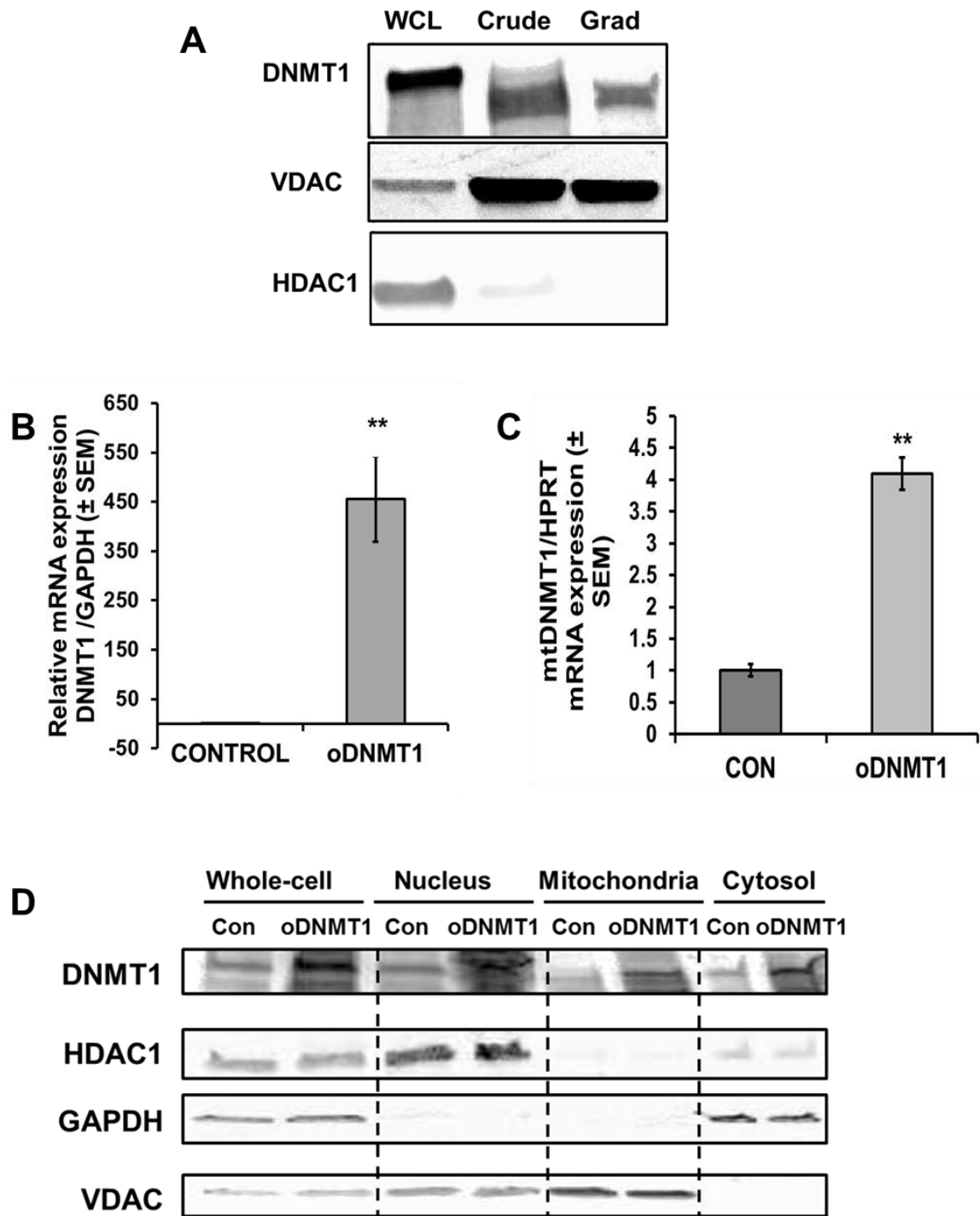
Several pathological conditions (Infantino, Castegna et al. 2011, Feng, Xiong et al. 2012, Pirola, Gianotti et al. 2013, Wong, Gertz et al. 2013, Zheng, Linarelli et al. 2015) and even physiological states (Ghosh, Sengupta et al. 2014) have been correlated with pattern of mtDNA methylation. However, evidences supporting such a causal relationship of mtDNA methylation are still lacking. Moreover, any association of mtDNA methylation with mitochondrial function is completely unexplored. We explored whether mitochondrial function is affected by alteration of mtDNA methylation by overexpression of DNMT1 and treatment with the DNA methylation inhibitor (5-aza-dC). Interestingly, it was found that both basal-mitochondrial oxygen consumption and total oxygen consumption decreased, irrespective of the trend (an increase or decrease) in mtDNA methylation and transcription of mtDNA-encoded genes. This tempted us to speculate that there may be a steady-state level of mtDNA methylation, which is required to maintain normal mitochondrial function and that deviation from such a steady-state may result in mitochondrial dysfunction. However, further mechanistic studies such as enzymes activity of the Electron Transport Chain (ETC), mtDNA-driven retrograde signaling along with determination of mitochondrial mass, are required to understand why a decrease in total and basal-mitochondrial oxygen consumption was observed in both the scenarios and how mitochondrial function is related to mtDNA methylation.

A limitation of our approach is that the overexpression of DNMT1 and 5-aza-dC treatment will affect the methylation levels in both the nuclear (Momparker, Cote et al. 1997, Biniszkiewicz, Gribnau et al. 2002, Momparker 2005) as well as the mitochondrial genome. An alternative for overexpression of DNMT1 could have been the overexpression of mitochondria-targeted DNMT1, but it is very difficult to control the level of overexpression and limit the non-specific localization of mitochondria-targeted-gene. Hence, we chose to overexpress DNMT1 instead of

the mitochondria-targeted-isoform, which is relatively a simpler approach. With regards to inhibiting DNA methylation, a technology to specifically inhibit methylation in mitochondria is lacking, which might have also hindered the field from pursuing mechanistic studies on mtDNA methylation. Irrespective of these limitations, since ours is one of the first studies to investigate role of mtDNA methylation in controlling transcription of mtDNA-encoded genes and subsequently mitochondrial function by attempting to alter mtDNA methylation, our results will pave foundation for further studies in this direction to get more valuable insights on functional relevance of mtDNA methylation.

In conclusion, our results strongly suggest mtDNA methylation to be an ongoing and alterable phenomenon. Along with alterations in mtDNA methylation by DNMT1 overexpression and 5-aza-dC treatment, concomitant changes in mitochondrial transcription and mitochondrial function were seen. Our results support the hypothesis that mtDNA methylation can regulate the transcription of mtDNA-encoded genes.

3.6 FIGURES



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Figure 1 Presence of DNMT1 in SH-SY5Y mitochondria and its global overexpression in SH-SY5Y neuroblastoma cell line: (A) Presence of DNMT1 was determined in the crude and gradient-purified mitochondria along with purity markers: mitochondrial marker (VDAC) and nuclear marker (HDAC1) with respect to the whole-cell lysate using western blot analysis. DNMT1 was overexpressed in SH-SY5Y cells and was confirmed by determining (B) its relative mRNA expression, (C) relative mRNA expression of mtDNMT1 and (D) the protein levels of DNMT1 in whole-cell, nuclear, mitochondrial and cytosolic fractions in cells that were transfected with the empty vector and the vector containing DNMT1. ** indicates a significant difference between groups at $p < 0.01$.

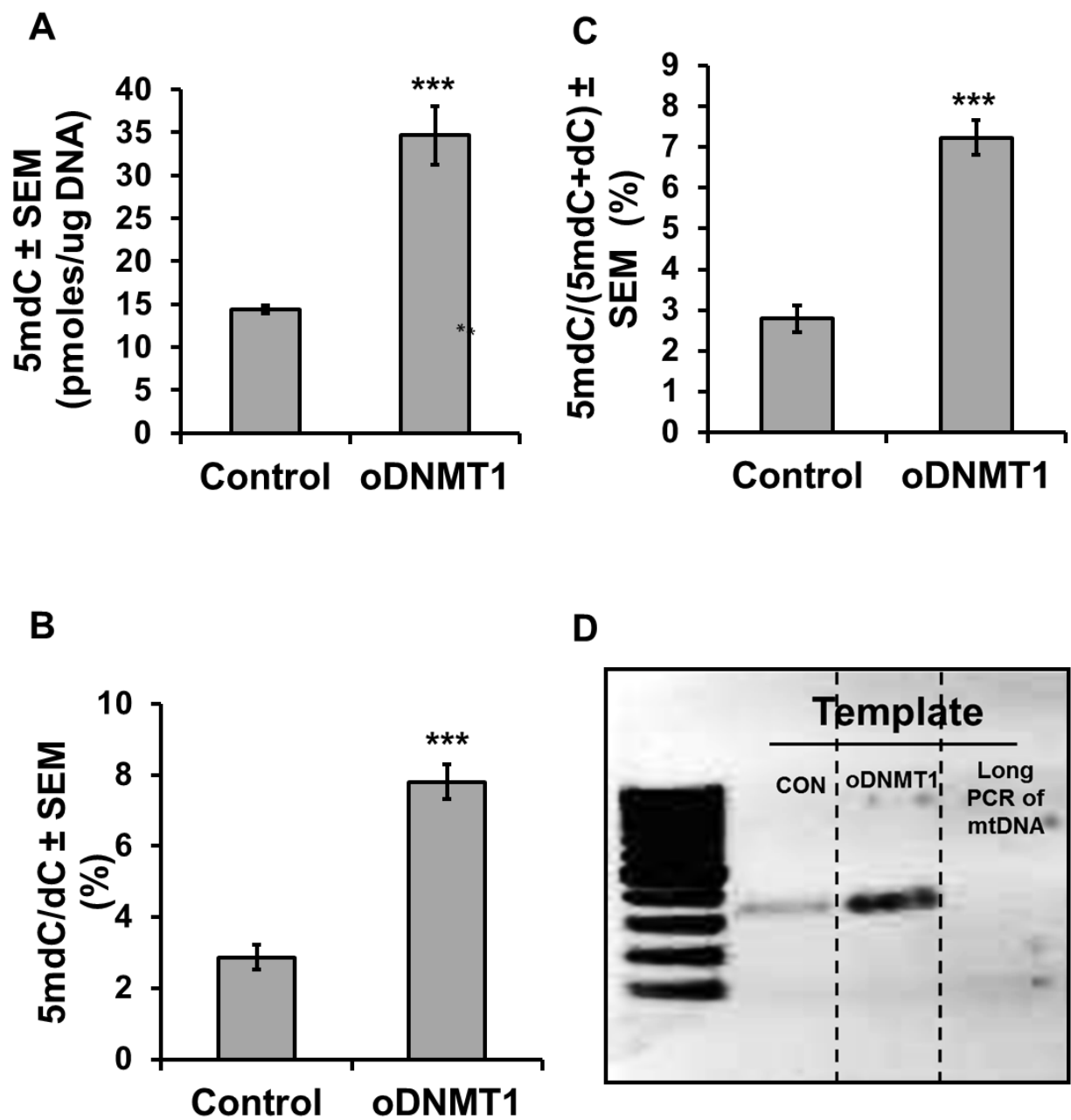
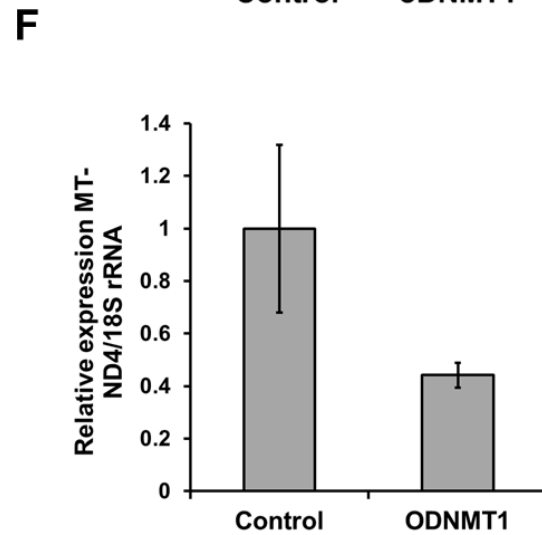
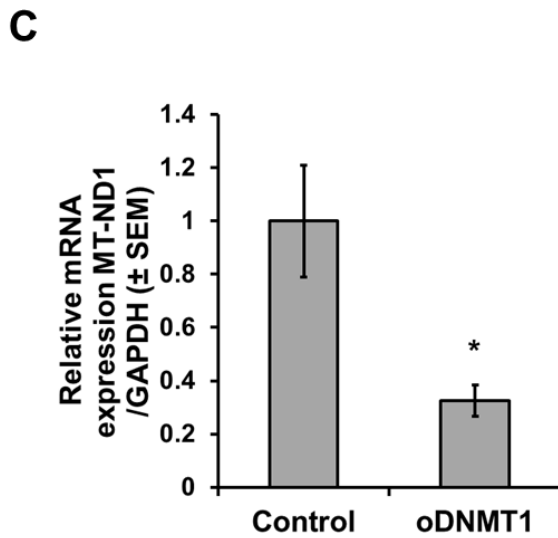
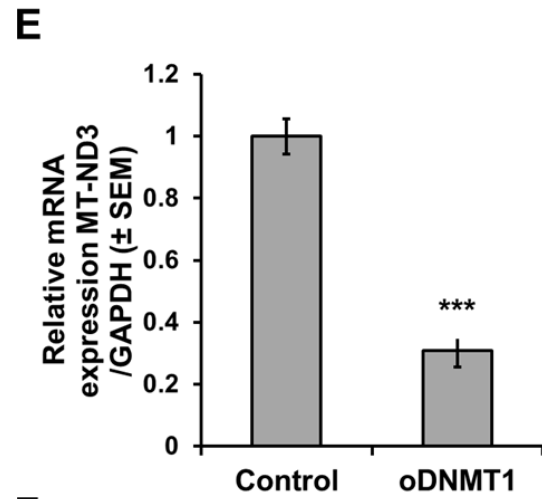
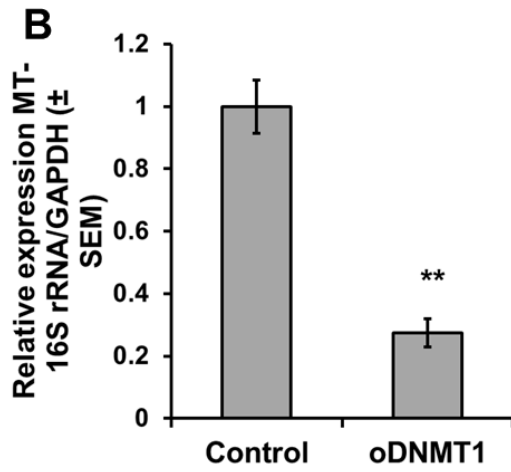
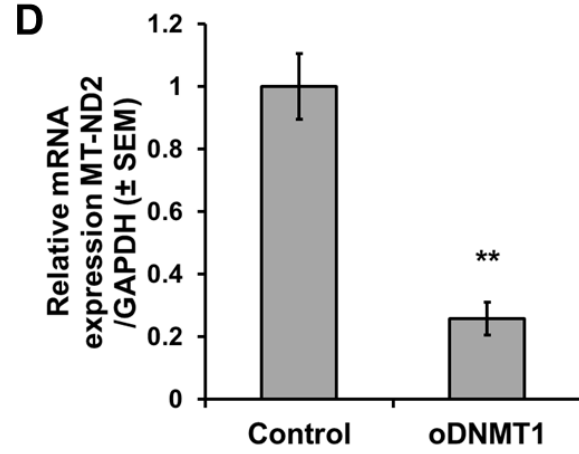
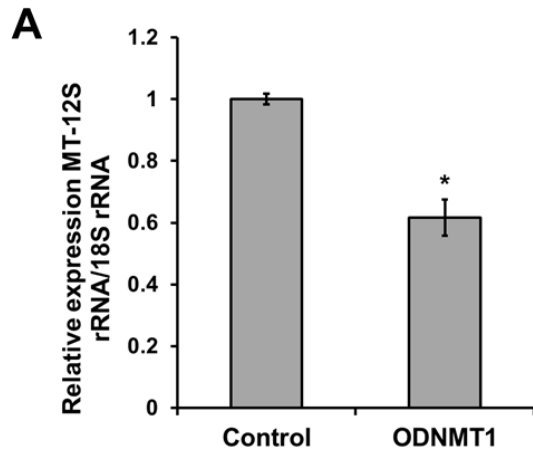
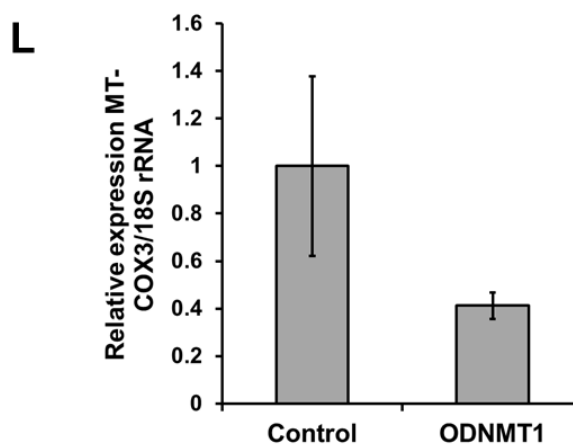
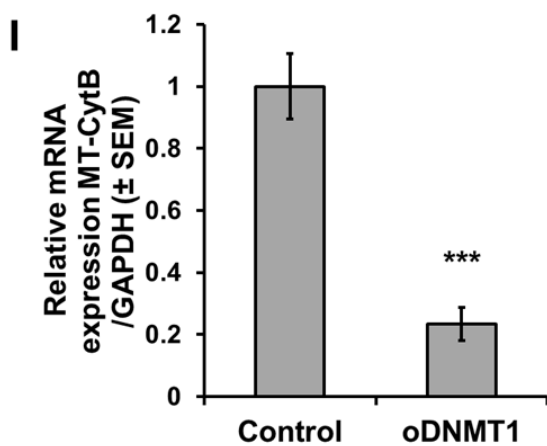
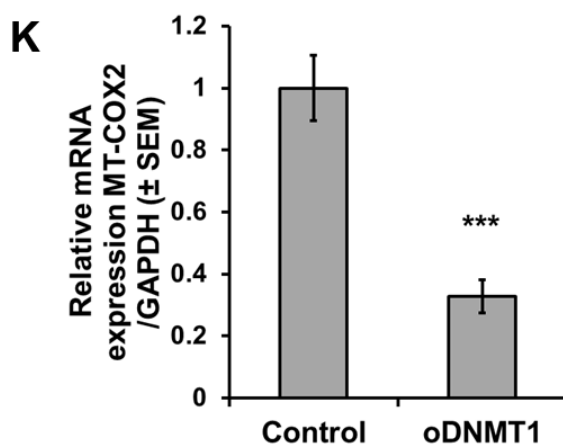
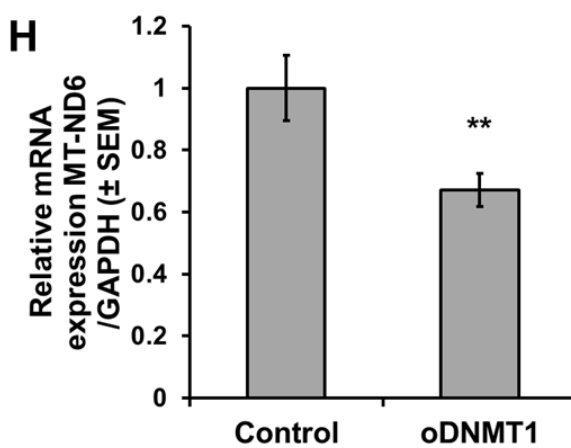
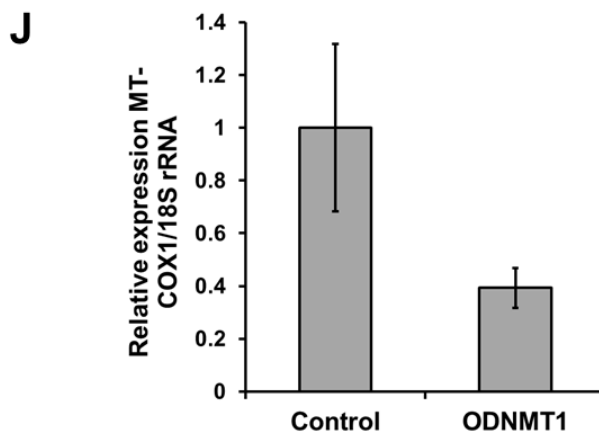
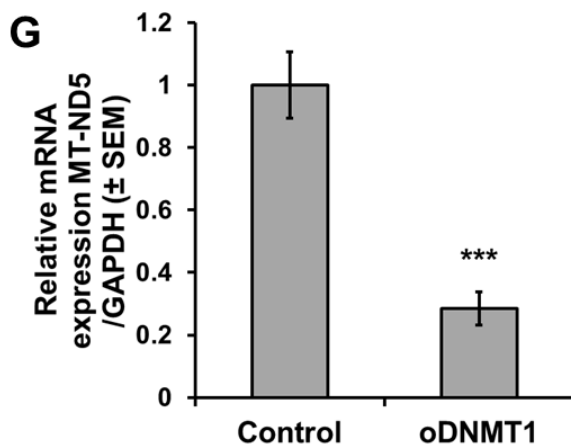


Figure 2 Overexpression of global DNMT1 increases mtDNA methylation: 5mdC was quantitated using HPLC-ESI-MS/MS in mtDNA extracted from cells that were transfected with the empty vector ‘control cells’ and the vector containing DNMT1 ‘oDNMT1 cells’, after they

were digested into nucleosides. 5mdC levels are represented as **(A)** absolute values (pmoles/ μ g DNA) and as percentages of **(B)** 5mdC/dC and **(C)** 5mdC/ (dC + 5mdC). **(D)** Methylation-specific primers for specific site in 12s rRNA were used to amplify from bisulfite-treated mtDNA prepared from ‘control cells’ and ‘oDNMT1 cells’; subsequent PCR products were electrophoresed through 1% agarose-gel containing ethidium bromide and visualized by UV as shown. *** indicates a significant difference between groups at $p < 0.001$.



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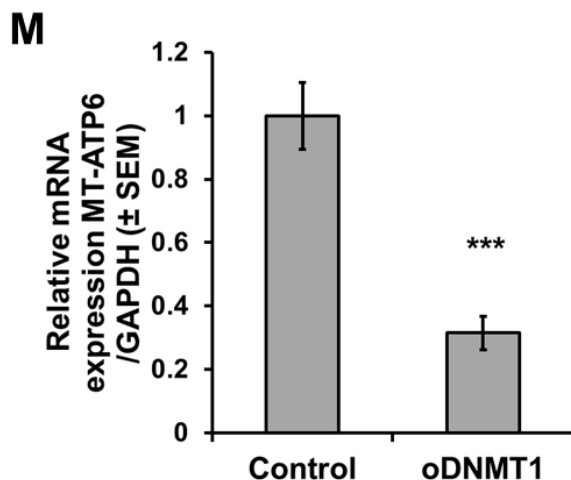
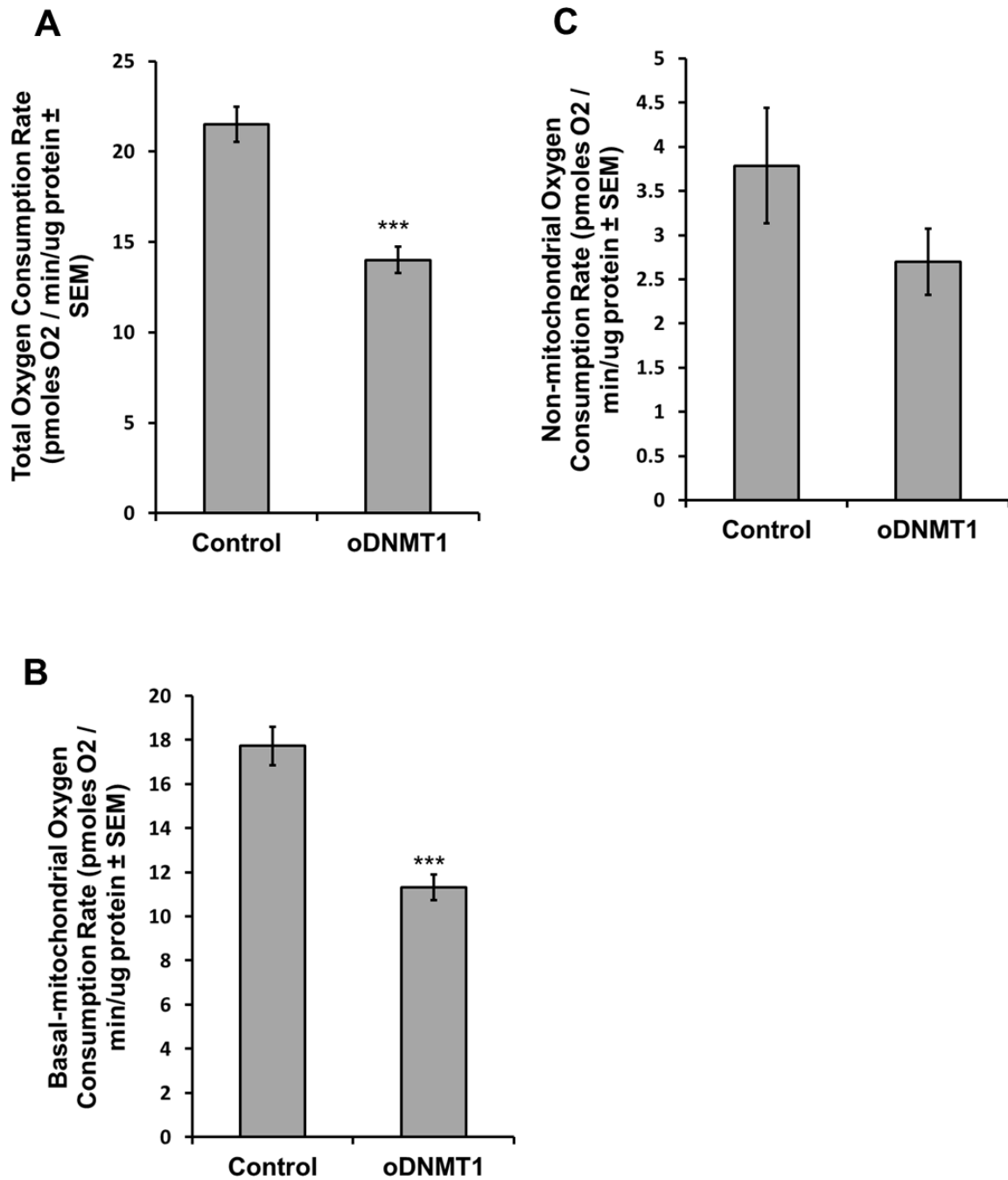
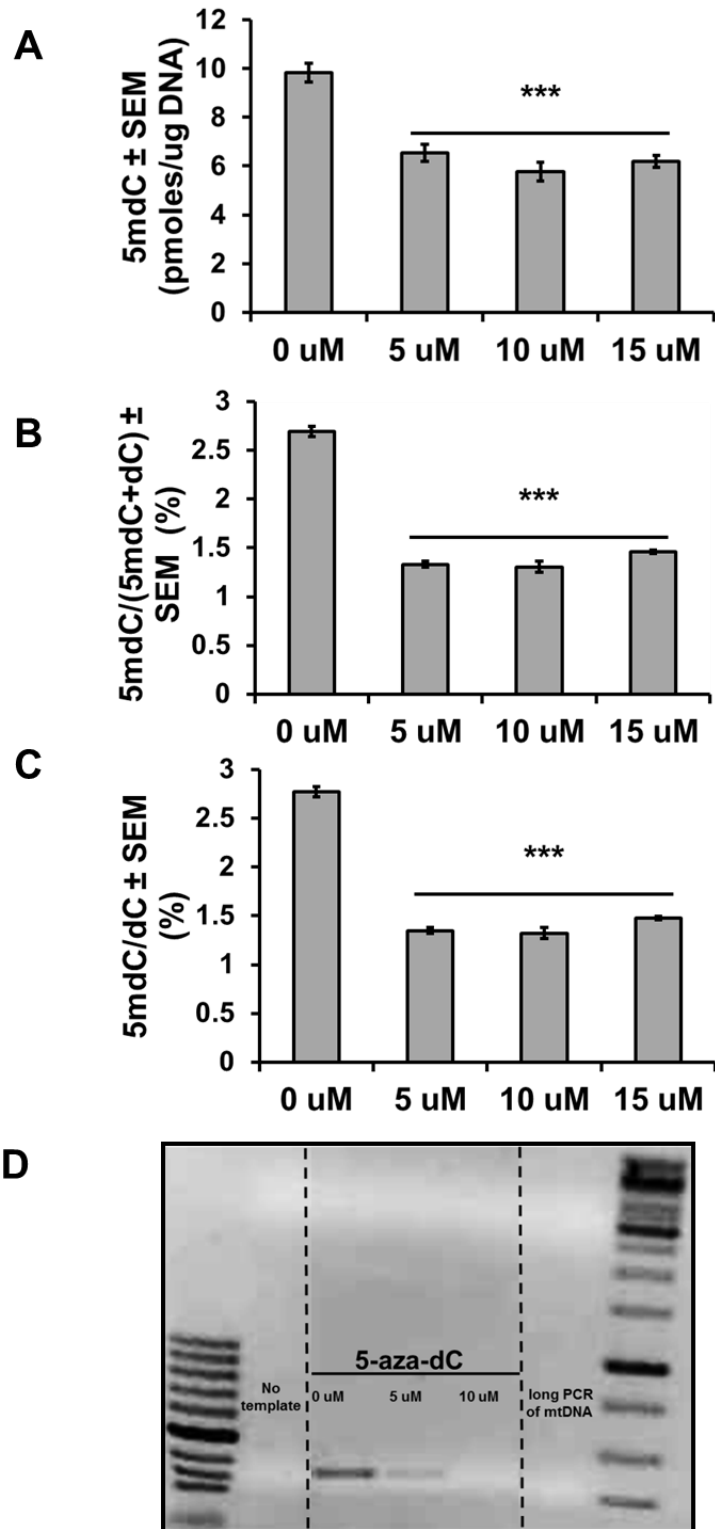


Figure 3 Overexpression of global DNMT1 decreases mtDNA transcription: Transcript levels of mtDNA-encoded genes were assayed for ‘control cells’ and ‘oDNMT1 cells’ by determining the relative expression levels of (A) MT-12S rRNA, (B) MT-16S rRNA, (C) MT-ND1, (D) MT-ND2, (E) MT-ND3, (F) MT-ND4, (G) MT-ND5, (H) MT-ND6, (I) MT-CytB , (J) MT-COX1, (K) MT-COX2, (L) MT-COX3 and (M) MT-ATP6. * indicates a significant difference between groups at $p < 0.05$. ** indicates a significant difference between groups at $p < 0.01$. *** indicates a significant difference between groups at $p < 0.001$.



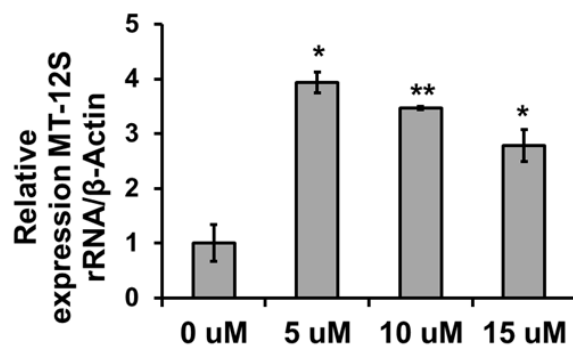
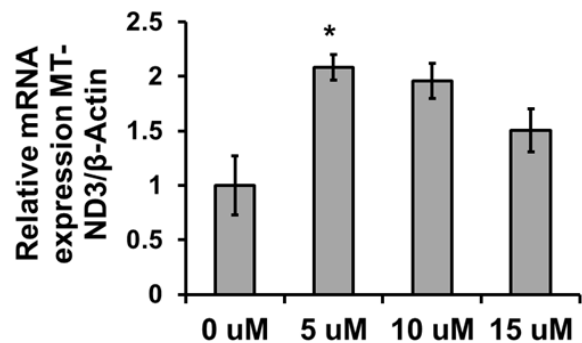
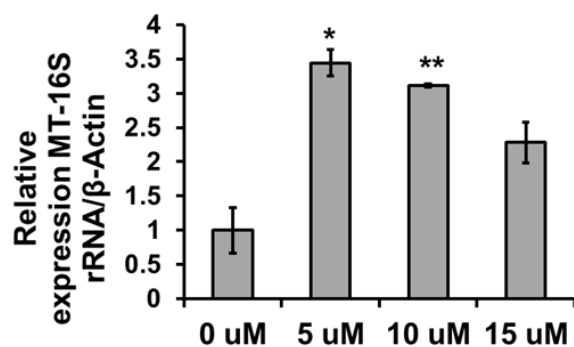
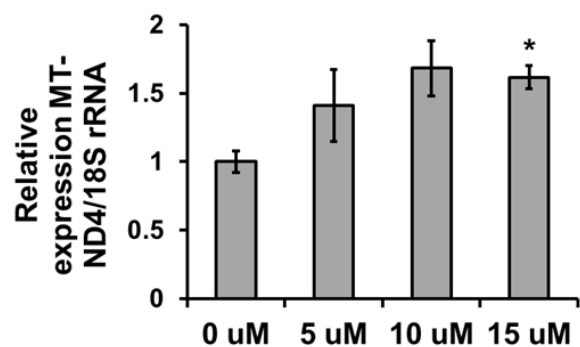
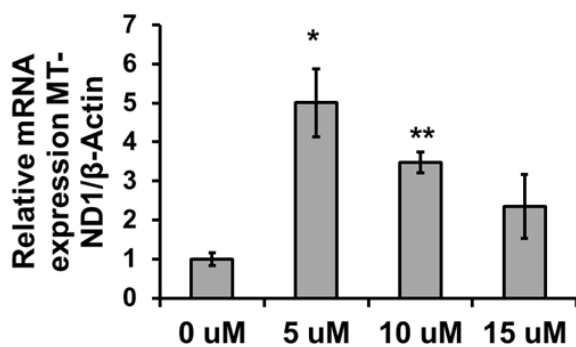
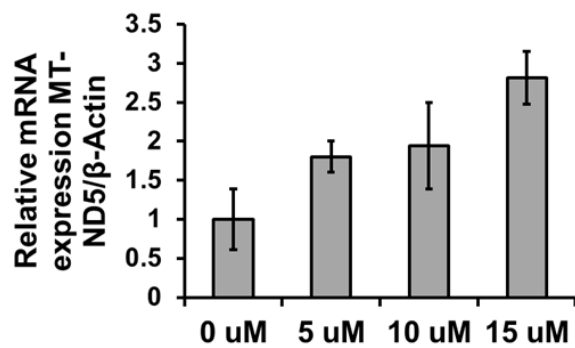
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Figure 4 Overexpression of global DNMT1 affects mitochondrial OCR: (A) Total OCR, (B) Basal- Mitochondrial OCR and (C) Non-Mitochondrial OCR were determined for ‘control cells’ and ‘oDNMT1 cells’. The units for all OCR (A-C) are pMoles/O₂/min/μg protein. *** indicates a significant difference between groups at p<0.001.

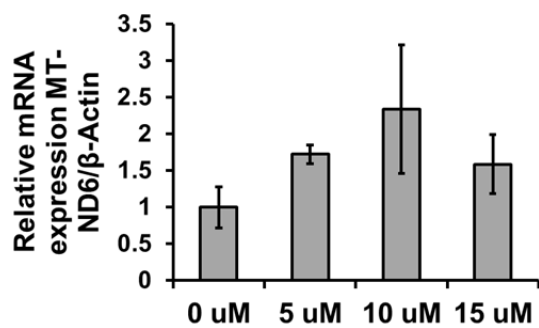
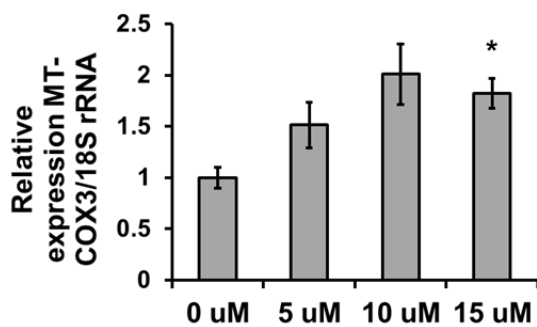
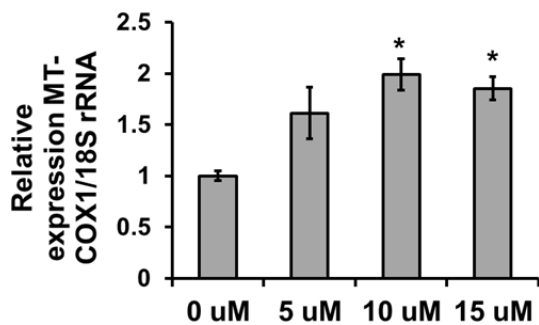
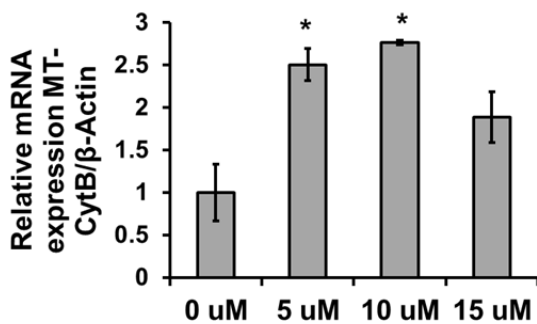
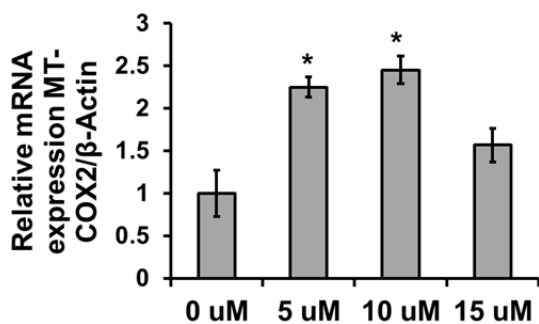
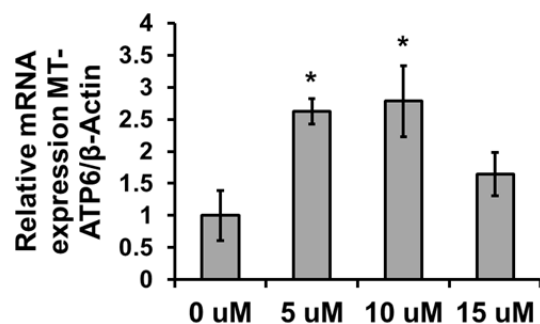


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Figure 5 DNA methylation inhibitor decreases mtDNA methylation: 5mdC was quantitated using HPLC-ESI-MS/MS in mtDNA extracted from cells that were treated with different concentrations of 5-aza-dC – 0 μ M, 5 μ M, 10 μ M and 15 μ M, after they were digested into nucleosides. 5mdC levels are represented as (A) absolute values (pmoles/ μ g DNA) and as percentages of (B) 5mdC/dC and (C) 5mdC/(dC + 5mdC). (D) Methylation-specific primers for specific site in 12s rRNA were used to amplify from bisulfite-treated mtDNA prepared from cells treated with different concentrations of 5-aza-dC – 0 μ M, 5 μ M and 10 μ M; subsequent PCR products were electrophoresed through 1% agarose-gel containing ethidium bromide and visualized by UV as shown. *** indicates a significant difference between groups at $p < 0.001$.

A**D****B****E****C****F**

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G**J****H****K****I****L**

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Figure 6 DNA methylation inhibitor increases mtDNA transcription: Transcript levels of mtDNA-encoded genes were assayed for cells that were treated with different concentrations of 5-aza-dC – 0 μ M, 5 μ M, 10 μ M and 15 μ M by determining the relative expression levels of (A) MT-12S rRNA, (B) MT-16S rRNA, (C) MT-ND1, (D) MT-ND3, (E) MT-ND4, (F) MT-ND5, (G) MT-ND6, (H) MT-COX1, (I) MT-COX2, (J) MT-COX3, (K) MT-CytB and (L) MT-ATP6

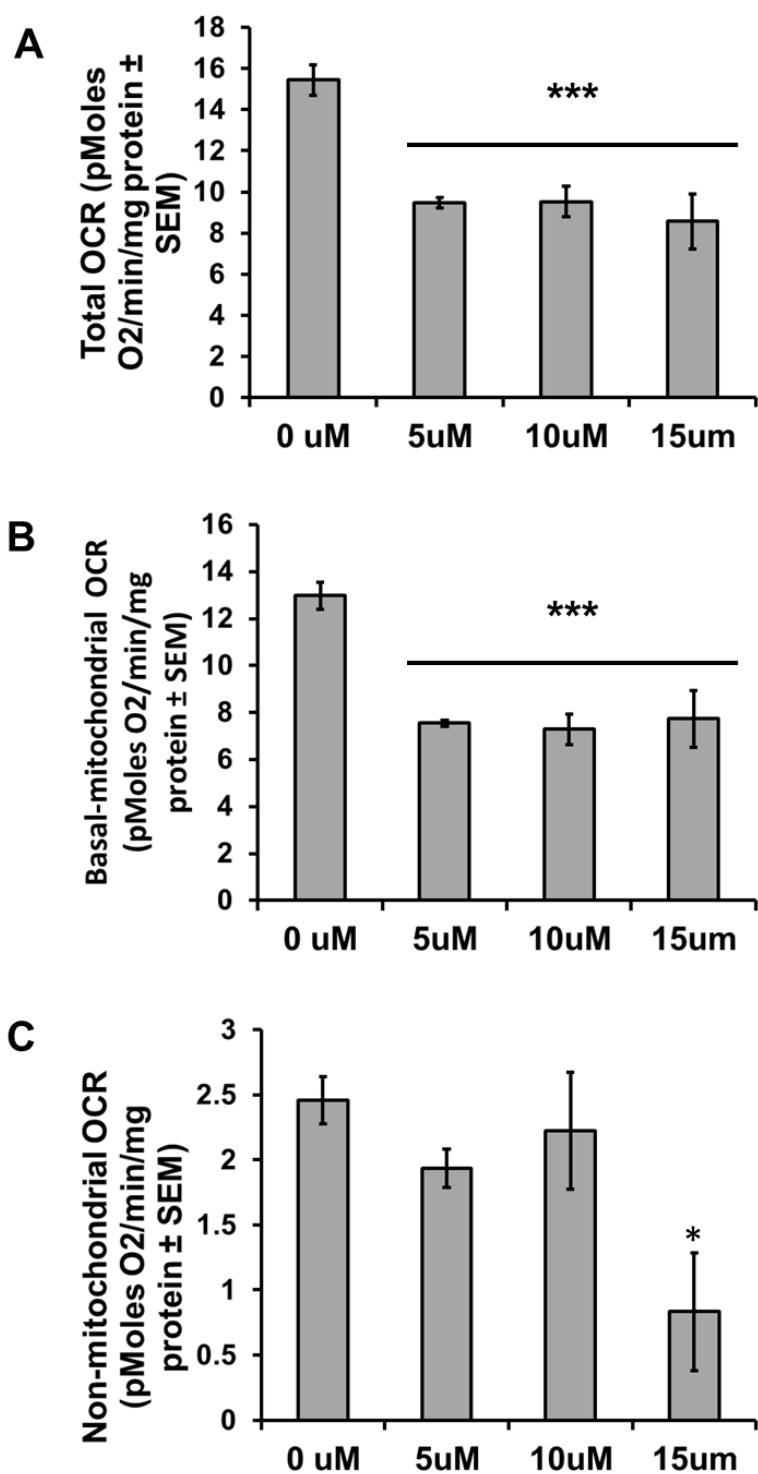


Figure 7: DNA methylation inhibitor affects mitochondrial OCR

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Figure 7 DNA methylation inhibitor affects mitochondrial OCR: (A) Total OCR, (B) Basal-Mitochondrial OCR and (C) Non-Mitochondrial OCR were determined for cells that were treated with different concentrations of 5-aza-dC – 0 μ M, 5 μ M, 10 μ M and 15 μ M. The units for all OCR (A-C) are pMoles/ O_2 /min/ μ g protein. *** indicates a significant difference with respect to 0 μ M group at $p < 0.001$.

CHAPTER IV: AGING-ASSOCIATED BIOENERGETICS, ITS REGULATORS AND
DNA METHYLATION IN A CYTOPLASMIC HYBRID MODEL CREATED FROM
PLATELETS OF YOUNG AND OLD HUMAN SUBJECTS

Nairita Roy, J. Eva Selfridge, Matt Stroh, Hao Zhu, Todd Williams, Eli Michaelis, Heather
Wilkins, Russell H. Swerdlow

4.1 ABSTRACT

Mitochondrial dysfunction is a hallmark of aging and age-related disorders. As mitochondria are bi-genomically encoded, mitochondrial function is governed by both the nuclear and mitochondrial genome (mtDNA). Although, alterations in mtDNA have been positively correlated with mitochondrial dysfunction during aging, direct role of mtDNA in aging-associated mitochondrial dysfunction has not been established. To investigate the role of human mtDNA in aging, we created cytoplasmic hybrid cell lines by transferring mitochondria from platelets of healthy young and aged human subjects to mtDNA-depleted SH-SY5Y cells. Bioenergetic fluxes, their regulators and DNA methylation-associated parameters were characterized in the resulting cytoplasmic hybrids. Relative to the cybrids generated from young donors, total oxygen consumption and basal-mitochondrial oxygen consumption were reduced in the cybrids made from aged subjects. Furthermore, COX activity was remarkably decreased in the cybrids created from aged subjects. Interestingly, there was a compensatory increase of Complex I activity in the cybrids generated from aged subjects. However, no alterations were found in ATP levels, redox states and glycolysis fluxes. Although the mitochondrial mass was not different, mRNA levels of some of the mtDNA-encoded OXPHOS genes and nuclear-encoded genes that promote mitochondrial biogenesis were induced in the cybrids created from aged subjects. As DNA methylation can regulate gene expression and also known to change in an age-dependent manner, DNA-methylation associated parameters were studied. Although total DNA methylation remained unaltered, DNMT1 protein, mRNA levels of mitochondrial DNMT1 and methylation in the mtDNA were higher in the cybrids generated from aged subjects, suggesting their role in age-related changes in bioenergetics. We conclude that age-dependent

changes in mtDNA have a direct role in determining bioenergetic flux, ETC function and expression of genes that control mitochondrial biogenesis.

4.2 INTRODUCTION

Mitochondrial dysfunction is known to occur during aging (Bratic and Larsson 2013), age-related diseases such as diabetes (Janssen, Maassen et al. 1999, van den Ouweland, Maechler et al. 1999), cardiovascular diseases (Tanaka, Konno et al. 1992), cancer (Chandra and Singh 2011) and neurodegenerative diseases such as Alzheimer's disease (Swerdlow 2011, Selfridge, E et al. 2013), Parkinson's disease (Cooper, Mann et al. 1992), Amyotrophic lateral sclerosis (Fujita, Yamauchi et al. 1996, Browne, Bowling et al. 1998) and encephalomyopathies (Holt, Harding et al. 1988, Wallace, Zheng et al. 1988, Holt, Harding et al. 1989). However, the mechanisms behind aging are poorly understood.

The primary function of mitochondria is to generate ATP through the process of oxidative phosphorylation (OXPHOS), performed by the Electron Transport Chain (ETC) consisting of Complex I-IV and ATP synthase (Complex V). Mitochondria contain their own genetic information in form of a circular double-stranded DNA called the mitochondrial DNA (mtDNA), which exists in multiple copies in a tissue-dependent manner. Mitochondrial proteins are encoded by both the nuclear genome as well as the mitochondrial genome. Along with 24 rRNAs/tRNAs that are required for mitochondrial translation, mtDNA encodes for 13 proteins required for ETC (except Complex II) and ATP synthase.

Aging-associated mitochondrial dysfunction and alterations in mtDNA have been observed in various organs in animal models (Vermulst, Wanagat et al. 2008) and clinical samples (Short, Bigelow et al. 2005). During aging, mtDNA mutations are known to accumulate (Trifunovic 2006), which are attributed to oxidative damage caused by elevated levels of reactive oxygen species (ROS) (Harman 1956, Harman 1981), the poor proof-reading efficiency of polymerase- γ

(Kaguni 2004, Trifunovic, Wredenberg et al. 2004, Pursell, McDonald et al. 2008, Vermulst, Wanagat et al. 2008) and also to the limited efficiency of the mtDNA repair machinery (Druzhyna, Wilson et al. 2008, Santos, Correia et al. 2013). Several studies have reported strong correlation of mtDNA mutations with decline in oxygen consumption and tissue-dependent decrease in enzymatic activities of ETC. However, there is lack of evidence for a direct role of mtDNA in aging-associated mitochondrial dysfunction (Bratic and Larsson 2013) due to concomitant control of mitochondrial function by both mtDNA as well nuclear DNA. This problem can be circumvented by using cytoplasmic hybrid (cybrid) system (Khan, Smigrodzki et al. 2007, Swerdlow 2007, Wilkins, Carl et al. 2014). Cytoplasmic hybrid (cybrid) cell lines can incorporate human subject's mitochondria and perpetuate mitochondrial DNA (mtDNA)-encoded components of the human subject. Since the nuclear background of different cybrid lines can be kept constant, this technique allows investigators to study the influence of mtDNA on various cellular functions. Prior usage of cybrids has elucidated the contribution of mtDNA to various parameters associated with mitochondrial function such as electron transport chain activities, bioenergetic fluxes, free radical production and mitophagy (Esteves, Domingues et al. 2008, Esteves, Lu et al. 2010, Swerdlow 2012, Silva, Selfridge et al. 2013).

Very few studies have addressed the direct role of mtDNA in aging-associated mitochondrial dysfunction using cybrid model. Mouse cybrid cell lines of different ages were created by fusing mitochondria from skeletal muscle, neurons and synaptosomes with mtDNA-depleted cell line and displayed age-related mitochondrial dysfunction (Li, Li et al. 2010, Li, Kumar Sharma et al. 2013). However, concerns have been raised previously, regarding the ability of mouse mtDNA to mimic human aging physiology (Demetrius 2006, Kazachkova, Ramos et al. 2013, Kowald and Kirkwood 2013). Moreover, very few studies have interrogated the direct role of human mtDNA

in aging-associated mitochondrial dysfunction and have arrived at conclusions contradictory to each other (Hayashi, Ohta et al. 1994, Laderman, Penny et al. 1996, Isobe, Ito et al. 1998). All these studies used human mtDNA from skin fibroblasts, but used different mtDNA-depleted cell lines and different model systems, namely – cytoplasmic hybrid (Hayashi, Ohta et al. 1994, Laderman, Penny et al. 1996) and nuclear hybrid (Isobe, Ito et al. 1998). While one group concluded that damage in the mtDNA was responsible for the observed differences in aging-dependent functional alterations of mitochondria (Laderman, Penny et al. 1996), the other group inferred that the nuclear genome is responsible for age-related respiration deficiency (Hayashi, Ohta et al. 1994, Isobe, Ito et al. 1998). Thus, a direct role of human mtDNA in age-associated mitochondrial dysfunction has not been established yet and further comprehensive studies are required in this regard.

Therefore, we wanted to determine if human mtDNA has a role in mitochondrial dysfunction observed during aging. We have created cybrid cell lines by fusing platelets from young and old human donors with mtDNA-depleted SH-SY5Y ρ^0 cells. We have found that age-dependent changes in mtDNA have a direct role in determining bioenergetic flux, ETC function and expression of genes controlling mitochondrial biogenesis.

4.3 MATERIALS AND METHODS

Human subjects

After obtaining informed consent, young ($n = 9$) and aged ($n = 7$) human subjects, who were healthy and without any neurodegenerative disease, underwent a 10 mL phlebotomy using tubes containing acid-citrate-dextrose as an anticoagulant. The average age of the young subjects who participated in this study was 29.1 ± 0.9 years, and that of the aged subjects was 75.1 ± 3.0 years

as represented in **Figure 1B**. The cybrids generated from young donors will be referred to as “Young Donors Cybrid or YDC” whereas the cybrids generated from aged donors will be referred to as “Aged Donors Cybrid or ADC”.

Creation of cybrid lines and the culture media

We used platelet mitochondria to generate cybrid cell lines from young and old subjects as demonstrated pictographically in **Figure 1A** and as described previously (Swerdlow, Parks et al. 1997). Briefly, warm histopaque was used to fractionate whole blood of the human donor for isolating platelets. Isolated platelets were washed with S-minimum essential medium (SMEM) and briefly triturated with SH-SY5Y $\rho 0$ cells in polyethylene glycol diluted with SMEM (Swerdlow, Parks et al. 1997). The products of this trituration were plated on T75 flasks, maintained for 1 week in $\rho 0$ growth medium, and then switched to cybrid selection medium for 6 weeks. SH-SY5Y $\rho 0$ cells lack intact mtDNA, do not possess a functional ETC, and are auxotrophic for pyruvate and uridine. Maintaining cells in the cybrid selection medium devoid of pyruvate and uridine, removes $\rho 0$ cells that have not acquired platelet mtDNA during trituration. Along with the true fusions, ‘mock fusions’ in which SH-SY5Y $\rho 0$ cells not triturated with platelets were plated and maintained in selection medium, and during the selection period all cells from the mock fusions died. After selection was complete, the resultant cybrid cells were changed to $\rho 0$ growth medium. Flasks were maintained in this medium at 37°C, 5% CO₂ for 24 h prior to harvesting. Thus, cybrid cell lines were made from 9 young donors and 7 aged healthy donors for this study.

Composition of the $\rho 0$ growth-media is DMEM containing 25 mM glucose, 10% FBS, 1% Penstrep, 100ug/mL pyruvate, 50ug/mL uridine whereas that of the cybrid selection-medium is

DMEM containing 25 mM glucose, 10% heat-inactivated-dialyzed FBS, 1% Penstrep, no pyruvate and no uridine.

Harvesting of cells

Cybrid cell lines growing in the culture flasks or petri-dishes were rinsed with 1X Phosphate Buffered Saline (PBS), trypsinized and then harvested using the p0 growth media. The harvested cells were then pelleted at 800g for 5 minutes at 4°C and subsequently washed with 1X PBS at 4°C for subsequent downstream procedure.

Oxygen consumption rate (OCR) & Extra-cellular acidification rate (ECAR) studies

Approximately 50,000 cells from each cybrid line were seeded in the wells of Seahorse XF cell culture microplates (Seahorse Bioscience, Billerica, MA) and standard two-step seeding procedure was performed as per the manufacturer's recommendations. The XF cell culture microplates were placed in a 37°, 5% CO₂ incubator for the cells to proliferate. The assays were done 48 hours post seeding.

OCR studies: Twenty four hours post seeding, the cells were starved off FBS into a buffered DMEM medium containing 5mM glucose. On the assay day (48 hours post seeding), before placing culture microplates in the Seahorse Analyzer the cells were washed with un-buffered DMEM containing 5mM glucose and 2 mM pyruvate and then placed in the same medium for an hour in an incubator without CO₂ at 37°C for degassing. OCR respiration measurements were collected after every cycle of 3 minutes of mixing, 2 minutes of wait time, followed by measurements for 3 minutes by the Seahorse Analyzer and the entire assay was completed in twelve such cycles as depicted in the following table.

Injection #	Cycles	Description
	1 st – 3 rd cycles	Total OCR was measured and 3 rd cycle reading was used for analysis.
1	3 rd – 6 th cycles	1 μ M Oligomycin was injected after the 3 rd cycle. The post-oligomycin OCR was measured and 6 th cycle reading was used for analysis.
2	7 th – 9 th cycles	0.3 μ M FCCP was injected after the 6 th cycle. The post-FCCP OCR was measured and 9 th cycle reading was used for analysis.
3	9 th – 12 th cycle	Mixture of 0.1 μ M Rotenone and 0.2 μ M Antimycin A was injected after the 9 th cycle. Non-mitochondrial OCR was measured and 12 th cycle reading was used for analysis.

The non-mitochondrial OCR was subtracted from the total OCR to yield the mitochondrial OCR.

The proton-leak OCR was obtained by subtracting the non-mitochondrial OCR from the post-oligomycin OCR whereas the maximal OCR was calculated by subtracting the non-mitochondrial OCR from the post-FCCP OCR.

ECAR studies: Twenty four hours post seeding, the cells were starved off FBS into a buffered DMEM medium containing no glucose. On the assay day (48 hours post seeding), before placing culture microplates in the Seahorse Analyzer, the cells were washed with un-buffered DMEM containing L-Glutamine and were placed in the same medium for an hour in an incubator without CO₂ at 37°C for degassing. The wells were read for ECAR after every cycle of 3 minutes of mixing, 2 minutes of wait period, followed by measurements for 3 minutes by the Seahorse Analyzer and the entire assay was completed in twelve such cycles as depicted in the following table.

Injection #	Cycles	Description
	1 st – 3 rd cycles	Basal ECAR was measured.
1	3 rd – 6 th cycles	20 mM glucose was injected after the 3 rd cycle. The post-glucose ECAR was measured and 6 th cycle reading was used for analysis.
2	7 th – 9 th cycles	1 mM Oligomycin was injected after the 6 th cycle. The post-Oligomycin ECAR was measured and 9 th cycle reading was used for analysis.
3	9 th – 12 th cycle	100 mM 2-deoxy-glucose was injected after the 9 th cycle. Non-Glycolytic ECAR was measured and 12 th cycle reading was used for analysis.

The non-glycolytic ECAR was subtracted from the post-glucose ECAR to provide the glycolysis ECAR. Next, the non-glycolytic ECAR was subtracted from the post-oligomycin ECAR to provide the glycolysis capacity ECAR. Subtracting the glycolysis ECAR from the glycolysis capacity ECAR yielded the glycolysis spare reserve capacity.

Both OCR and ECAR studies were repeated at least three times. The readings obtained for the particular day were normalized by the mean of ADC for a particular parameter and averaged across multiple days before statistical analysis to yield the relative values of each parameter.

Isolation of mitochondria

Mitochondria were isolated as described previously (Kristian, Hopkins et al. 2006) with some modifications. Briefly, cells growing in a confluent T180 flask of each cybrid cell line were harvested as described above. The washed cell pellets were then re-suspended in MSHE buffer, collected in a pre-cooled cavitation chamber (nitrogen bomb; Parr Instrument Company, Moline,

IL) and subjected to 900 psi for 15 minutes. The cell suspension is collected by releasing the pressure instantaneously and centrifuged at 1000g for 5 minutes at 4°C to pellet the cell debris. The supernatant was collected and spun at high speed of 20000g for 10 minutes at 4°C. The pellet obtained was re-suspended in MSHE and is the crude preparation of mitochondria and used for COX assay.

Mitochondria were also isolated using Mitochondria Isolation Kit for Cultured Cells (Abcam 110170) for Complex I assay and isolation of mitochondrial DNA. Briefly, cells growing in a confluent T180 flask of each cybrid cell line were harvested as described above. The cell pellet was then freeze-thawed to weaken the cell membranes, suspended in Reagent A and kept on ice for 10 minutes. The suspension was then homogenized and spun at 1000 g for 10 minutes at 4°C, to pellet the cellular debris and collect the supernatant that contains the mitochondria. The cellular debris were re-suspended in Reagent B, incubated on ice for 10 minutes again, dounce homogenized and spun again to collect the supernatant that contains the mitochondria. Both the supernatants were combined and centrifuged at 12000 g for 15 minutes at 4°C to pellet the mitochondria.

Cytochrome c oxidase (COX) assay and Citrate Synthase assay

COX assays were performed on isolated mitochondria and whole cell pellets whereas citrate synthase assays were performed on whole cell pellets only as described previously (Ghosh, Patel et al. 2007). After the assay was performed, protein concentration was determined by DC Protein Assay (Biorad, Hercules, California, USA). The rate obtained was then corrected for the amount of protein in the cuvette.

Complex I activity-ELISA based assay`

Complex I activity was assayed using Complex I Enzyme Activity Microplate Assay Kit (Abcam 109721). Briefly, the isolated mitochondria were permeabilized using the detergent and the enzyme Complex I, is immuno-captured within the wells of the microplate and its activity is determined by following the oxidation of NADH to NAD⁺ and the simultaneous reduction of a dye which leads to increased absorbance at 450 nm. Thus, the rate constant (V_{max}) in min⁻¹ was spectrophotometrically determined. Each cybrids line's rate was then normalized to the amount of protein in the mitochondrial preparation and divided by the extinction coefficient of the dye, which is 25.9 mM⁻¹.

ADP/ATP assay

ADP levels, ATP levels and the ADP/ATP ratios were determined using the EnzyLight™ ADP/ATP Ratio Assay Kit (Bioassay Systems, CA, USA). Briefly, 6x10⁵ cells per well were seeded in 96 well plates and 48 hours later the assay was performed. In the first step, cells were lysed to release ATP and ADP. ATP reacts with the substrate D-Luciferin in presence of the enzyme luciferase, and the subsequent fluorescence reaction provides a direct indication of the ATP concentration. Next, the change in fluorescence reaction was followed as ADP was converted to ATP. The difference between the initial and final fluorescence indicated the ADP concentration.

NAD⁺/NADH assay

Cells were harvested and assayed for NAD⁺/NADH levels using the Fluorescent NAD/NADH Detection Kit (Cell Technology, Inc., CA, USA). Briefly, 2x10⁶ cells were seeded in 60 mm dish, harvested after 48 hours and divided into two different vials, one for NAD⁺ and another for NADH. Cells were then re-suspended in the appropriate extraction and lysis buffers. Cell suspensions were then vortexed for homogenization and the lysates were incubated at 60°C for

15 minutes. These lysates were cooled immediately and reaction buffer, along with the opposite buffer for neutralization was added. Samples were centrifuged at 8000 g for 5 minutes to clarify the supernatants. The supernatants obtained were then assayed for NAD⁺/NADH with the following fluorescence measurements - excitation 530 nm and emission 590 nm.

DNA extraction

DNA extraction was done by the method based on organic-solvent extraction of DNA as described previously (Guo, Jiang et al. 2009). Briefly, the sample in ice-cold lysis buffer underwent dounce homogenization, followed by Proteinase K digestion at 55°C for 3h in a water bath. The mixture was spun to remove the non-soluble fractions and the DNA in the supernatant was extracted by standard Phenol-Chloroform-Isoamyl alcohol method. DNA was precipitated using Sodium Acetate and isopropanol at -20°C. The DNA pellet was washed twice with 70% ethanol. Finally, the extracted DNA pellet is then air-dried, re-suspended in nuclease-free water and then quantitated using Infinite M200 (Tecan, San Jose, USA).

Total DNA was extracted for mtDNA copy number assay and to determine the amount of global methylation. Two million cells from each cybrid line were seeded in 60 mm dish and harvested 48 hours later as described above and the washed cell pellets were suspended in ice-cold Lysis buffer for DNA extraction as described above.

The mitochondrial DNA was extracted for determining the amount of mtDNA methylation for every cybrid line. Twenty million cells of every cybrid cell line were seeded in a T180 flask and harvested 48 hours later. Cells were harvested and the mitochondria were isolated from every cybrid line as described above. The isolated mitochondria were suspended in ice-cold Lysis buffer for DNA extraction as described above.

RNA extraction

Two million cells from each cybrid line were seeded in 60 mm dish and harvested 48 hours later. RNA extraction was performed using TRI Reagent solution (Life technologies AM9738). Briefly, supernatant was removed and TRI was added to harvest the cells. The homogenate was incubated at room temperature to allow disassociation of the nucleoprotein complexes. Chloroform was used to separate the protein from the nucleic acids. RNA was precipitated using isopropanol; the pellet obtained was washed with 70% ethanol and then air-dried before re-suspending in nuclease-free water. The extracted RNA is then further quantitated using Infinite M200 (Tecan, San Jose, USA).

cDNA synthesis

1 µg of RNA was used from every cybrid line to synthesize cDNA using iScript Reverse transcription 5X super-mix in a 20 µL reaction as per the recommendations in the protocol (Biorad 170-8840). The 20 µL reaction was then diluted 5 times by adding 80 uL nuclease-free water. 2 µL of the diluted cDNA solution was used for every qPCR reaction.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and ready-to-use TaqMan Gene Expression Assays (Applied Biosystems). We quantified mRNA levels deriving from the following genes: PGC1 α , PGC1 β , PRC, NRF1, NRF2, TFAM, MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND5, MT-ND6, MT-COX1, MT-COX2, MT-COX4Ii, MT-CytB and MT-ATP6. GAPDH was used as an internal loading control. qPCR amplification was determined utilizing an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems).

To quantify mtDNA by mtDNA copy number assay, we used the TaqMan Gene Expression Assay Kit (Applied Biosystems) and primers for three mtDNA-encoded genes, 16S RNA gene,

NADH dehydrogenase subunit 2 (MT-ND2) and Cytochrome c oxidase 1 (MT-COX1), as well as to the nuclear 18S rRNA gene. The primers were used to amplify from 5 ng of total DNA in every PCR reaction. Relative ratios of mtDNA to nuclear DNA is called the mtDNA copy number and was determined using the comparative $\Delta\Delta CT$ method, and thus 16S/18S, MT-ND2/18S and MT-COX1/18S ratios were calculated.

Immunoblotting

To check for total protein levels, whole cell lysates were prepared by M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Protein concentrations for all the lysates were determined using a BCA Protein Assay kit (BioRad). Equal amount of proteins from the protein lysates of every cybrid line were resolved by electrophoresis in pre-cast 4–12% gels (BioRad), and then transferred to polyvinyl di-fluoride (PVDF) membranes. Non-specific binding was blocked by gently agitating the membranes in 5% non-fat milk and 0.1% Tween in PBS for 1 hour at room temperature. Blots were subsequently incubated in 5% bovine serum albumin (BSA) containing 0.1% Tween with the designated primary antibody overnight at 4°C with gentle agitation. Blots were washed thrice for 10 minutes with PBS containing 0.1% Tween three times and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature with gentle agitation. After three washes, the blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Chemiluminescence signals were detected using a Bio-Rad ChemiDoc Imager and band densities determined using Quantity One Software. Primary antibody for DNMT1 was purchased from Cell Signaling Technology (Beverly, MA, USA) and that of PGC1 α was purchased from Molecular Probes (Molecular Probes, Eugene, OR, USA). Both of them were used at a 1:1000 dilution.

HPLC-ESI-MS/MS assay

MtDNA was extracted followed by isolation of mitochondria from every cybrid line as described above. 1 µg of mtDNA in 50 µL of digestion buffer was incubated at 37°C overnight as described previously (Quinlivan and Gregory 2008) and the digestion products for every cybrid line was quantitated by HPLC-ESI-MS/MS. The digestion buffer consists of 2.5U benzonase, 3 mU of alkaline-phosphatase and 2 U phosphodiesterase-I in 20 mM Tris-HCl pH 7.9 containing 100mM NaCl and 20mM MgCl₂, which digests the mtDNA into nucleosides. Pmoles of 5-methyl-deoxy-cytidine (5mdC) present per ug of mtDNA were compared. To account for inter-sample variability in digestion, 5mdC (pmoles/ug DNA) were normalized by with the amount of deoxy-Cytidine (dC) and sum of dC and 5mdC (dC + 5mdC), to yield percentages and then compared between the group of cybrids.

Data analysis

Data are expressed as means ± standard error of the means (SEM). To compare means between two groups we used two-way, unpaired Student's t-tests, with p-values of <0.05 considered significant.

4.4 RESULTS

Reduced Total and Mitochondrial Oxygen Consumption Rate (OCR) in the cybrids created from aged donors

We used Seahorse XF Analyzer to determine oxygen consumption rates (OCR) in the cybrids generated from young and aged donors in unstressed assay conditions of 5mM glucose and 2 mM pyruvate. At the end of the assay, rotenone (a Complex I inhibitor) and Antimycin (a Complex III inhibitor) was injected that blocked the mitochondrial respiration, and thus provide the non-mitochondrial OCR. The basal mitochondrial OCR was obtained by subtracting the non-

mitochondrial OCR from the total OCR. Under unstressed conditions, cybrids made from aged donors had significantly lower total OCR (37% decrease) than the cybrids made from young donors (**Figure 2A**). Further, the basal-mitochondrial OCR was significantly decreased (45% decrease) in the cybrids made from aged donors when compared to the cybrids made from young donors (**Figure 2B**). However, the non-mitochondrial OCR were similar between the cybrids made from young and aged donors (**Figure 2C**), suggesting that changes in total OCR were due to alteration in basal-mitochondrial OCR only. These results indicate that age-dependent alterations in mtDNA play a role in decreasing oxygen consumption observed during aging-associated mitochondrial dysfunction.

Oligomycin is an inhibitor of ATP synthase. Oxygen consumption rates due to ATP production and proton-leak were measured post-oligomycin injection of 1 μM and estimated after subtracting the non-mitochondrial OCR, but were found to be comparable between the cybrids made from young and aged donors (data not shown). FCCP is a protonophore (H^+ ionophore) and can thus change the location of protons across inner-mitochondrial membrane. By bringing all the protons to the matrix, FCCP can force the mitochondria to consume oxygen at the highest rate possible, also called the maximal OCR. Maximal oxygen consumption capacity rates were measured post-FCCP injection of 0.3 μM and estimated after subtracting the non-mitochondrial oxygen consumption rate, but were found to be not different between the cybrids made from young and aged donors (data not shown).

No alteration in Glycolysis in the cybrids created from aged donors when compared to cybrids made from young donors

XF Seahorse Analyzers can also measure pH of the medium and thus can determine the rate at which cells acidify their surrounding medium. Since lactic acid excretion by the cultured cells is

primarily responsible for acidification of the medium, and lactic acid is produced by anaerobic glycolysis, Extra-Cellular Acidification Rate (ECAR) can be used to estimate glycolysis fluxes. Cells from each of our cybrid lines were placed in a glucose-free medium so that the initial ECAR values were not only quite low but also equivalent between the groups. 2-Deoxy-D-glucose is a modified glucose molecule in which the 2-hydroxyl group is replaced by hydrogen and thus inhibits further glycolysis. At the end of the experiment, 2-Deoxy-D-glucose was added to every well, and this revealed that the baseline ECAR measurements were stable throughout. The post-2-deoxyglucose ECARs were used to provide non-glycolysis acidification rates. Next, cells were subjected to glucose, with a final concentration of 20 mM. The non-glycolysis acidification rate for each line was subtracted from the rate in 20 mM glucose to yield the true glycolysis-dependent ECAR or the glycolysis rate. No significant difference was found in glycolysis rate (**Figure 2D**) between the cybrids made from young and aged donors. The glucose injection was followed by an injection of oligomycin with a final concentration of 1 mM that eliminates mitochondrial ATP production by inhibiting the mitochondrial ATP synthase and forces a compensatory increase in glycolysis-related ATP production. On subtracting the non-glycolysis acidification rate from the post-oligomycin ECAR, glycolysis capacity was obtained. We found that the glycolysis capacity was similar between the cybrids made from young and aged donors (**Figure 2E**). The post-glucose, pre-oligomycin ECAR was subtracted from post-oligomycin ECAR, to provide the glycolysis spare reserve capacity, which was again found to be not different between two groups (**Figure 2F**).

Reduction in Cytochrome c oxidase (COX or Complex IV) activity in the cybrids created from aged donors

Complex IV activity is known to decrease upon aging (Boffoli, Scacco et al. 1994, Ojaimi, Masters et al. 1999). Since we found a significant reduction in basal-mitochondrial OCR in the cybrids generated from aged donors, we investigated if the COX activity is altered. The isolated mitochondria and whole cells from each of the cybrid lines were assayed for the Cytochrome c oxidase activity. The Mito-prep COX activities were derived by normalizing the V_{\max} activities obtained from the isolated mitochondria by the amount of mitochondrial protein for each cybrid line. It was found that the Mito-prep COX activity was significantly reduced in the cybrids made from aged donors (**Figure 3A**). Similarly, the Whole-Cell COX activities were derived by normalizing the V_{\max} activities obtained from the whole cells by the amount of total protein in whole-cells extract for each cybrid line. The whole-cell COX activity was also found to be significantly reduced (by 32%) in the cybrids made from aged donors (**Figure 3B**). The whole-cell COX activities were further normalized for the amount of mitochondrial protein by assaying the activity of citrate synthase, an enzyme located in the mitochondrial-matrix, for every cybrid line. The resultant COX activity/CS activity was also found to be significantly reduced (by 26%) in cybrids made from aged donors (**Figure 3C**). These results clearly indicate that in comparison to the cybrids made from young donors, cytochrome c oxidase activity was impaired in the cybrids made from aged donors and was influenced by age-dependent alterations in mtDNA.

Increase of Complex I activity in the cybrids created from aged donors

Next, we investigated if the Complex I activity is altered between cybrids made from young and aged donors. Isolated mitochondria from each of the cybrid lines were assayed for the Complex I activity. The Complex I activity were normalized for the amount of protein in the isolated mitochondria for each cybrid line. Although the Complex I activity trended to be higher (*p value*: 0.0517) in the cybrids made from aged donors than that of the young donors, the difference was

not statistically significant (**Figure 3D**). However, when the Complex I activity of each cybrid line were further normalized using their respective citrate synthase activity, the cybrids made from aged donors were found to have significantly higher ratio of Complex I activity/Citrate Synthase activity (**Figure 3E**).

Similar levels of mitochondrial mass markers in the cybrids created from young and aged donors

As we observed differences in basal-mitochondrial OCR, complex IV activity and complex I activity, it was important to determine if differences in mitochondrial mass were responsible for these changes. To evaluate the mitochondrial mass, mtDNA copy number and citrate synthase activity assays were performed for every cybrid line.

For mtDNA copy number assay, we determined the relative mtDNA to nuclear DNA ratios in our cybrid lines. The copy number of the mtDNA-encoded genes were normalized to the copy number of nuclear gene 18S rRNA. No significant differences were found in the ratios of 16S rRNA: 18S rRNA (**Figure 4A**), MT-ND2:18S rRNA (**Figure 4B**) and MT-COX1: 18S rRNA (**Figure 4C**) between the cybrids created from young and aged donors.

As a mitochondrial enzyme, the citrate synthase can be used as a normalization factor for mitochondrial protein as well as a biomarker for mitochondrial content of a cell. Even the citrate synthase activity was found to be comparable between the two groups of cybrids (**Figure 4D**). These results indicate that the observed differences between the young and old group of cybrids were not due to differences in mitochondrial content or mass.

No alteration in energy and redox intermediates in the cybrids created from young and aged donors

Levels of ATP (**Figure 5A**) and the ratio of ADP: ATP (**Figure 5B**) were determined for every cybrid line from both the groups of cybrids, but no significant alteration was found between the cybrids made from young and aged donors. Levels of NAD⁺ (**Figure 5C**) and NADH (**Figure 5D**) were determined for every cybrid line from both the groups of cybrids and were also found to be comparable between the two groups of cybrids. The levels of NAD⁺ were normalized for the levels of NADH for every cybrid line to yield the NAD⁺: NADH ratios, which again were found to be similar between cybrids made from young and aged donors (**Figure 5E**).

Induction of transcriptional co-activators and transcription factors that promote mitochondrial biogenesis in the cybrids created from aged donors

PPAR γ co-activator-1 (PGC-1) family of transcriptional co-activators consists of PGC-1 α , PGC-1 β and PGC-related coactivator (PRC); plays a critical role in the regulation of mitochondrial oxidative metabolism and maintenance of glucose, lipid and energy homeostasis. PGC-1 α is vastly studied and its protein levels are known to decrease upon aging. Hence total PGC-1 α protein levels were checked, but were found to be similar between the cybrids made from young and aged donors (**Figure 6A-B**). Relative mRNA levels of PGC-1 α were not different either between the two groups of cybrids (**Figure 6C**). However, relative mRNA levels of other members of PGC-1 family, PGC-1 β and PRC were assayed and PGC-1 β trended to be higher in the cybrids made from aged donors whereas PRC was found to be significantly higher in the cybrids made from aged donors than that of young donors (**Figure 6D-E**).

PGC-1 family promotes mitochondrial biogenesis by activating the transcription factors that regulate the expression of nuclear-DNA-encoded mitochondrial proteins. These transcription factors include NRF1 and NRF2. Relative mRNA levels of NRF1 (**Figure 6F**) and NRF2 (**Figure 6G**) were assayed and were found to be significantly elevated in the cybrids made from

aged donors. The activation of these transcription factors increases the expression of many mitochondrial proteins, including mitochondrial transcription factor A (TFAM). TFAM stimulates transcription of mtDNA by binding to the promoters in mtDNA. Thus, by regulating TFAM levels, PGC-1 coactivators can influence the expression of proteins encoded by mtDNA. TFAM mRNA levels were investigated and found to be induced 2.8 fold times in cybrids made from aged donors when compared to cybrids made from young donors (**Figure 6H**).

These results indicate that age-associated differences in mtDNA can alter the expression of nuclear-encoded genes involved in mitochondrial biogenesis, which can be a compensatory phenomenon to mitigate the reduced mitochondrial function in the cybrids created from aged donors.

Alteration of mRNA levels of mtDNA-encoded OXPHOS genes in the cybrids created from young and aged donors

The process by which the proton-gradient is created across the inner-mitochondrial membrane and utilized for synthesis of ATP is called oxidative phosphorylation (OXPHOS). The OXPHOS apparatus located in the mitochondria is bi-genomically encoded, i.e. it is encoded by two genomes- the nuclear genome and the mitochondrial genome (mtDNA). MtDNA encodes for 2 rRNAs, 22 tRNAs and 13 mRNAs and all these 37 genes need to be transcribed for the mitochondria to function. The translation products of 13 mRNAs from the mtDNA, along with the nuclear-encoded OXPHOS proteins, form the functional subunits of different complexes (Complex-I and Complex-III-V) in the OXPHOS apparatus. As mRNA levels of transcriptional co-activators (**Figure 6D-E**) and transcription factors (**Figure 6F-H**) that promote mitochondrial biogenesis, especially TFAM were elevated in the cybrids created from aged donors, we studied if transcription of mtDNA-encoded OXPHOS genes was different, which can be an important

determinant of mitochondrial function. Relative mRNA levels of MT-ND1 (**Figure 7A**) and MT-ND5 (**Figure 7E**) that encode for Complex I components were found to be significantly higher in the cybrids made from aged donors. Relative mRNA levels of other mtDNA-encoded genes (**Figure 7B-D, F-J**) were slightly higher in the cybrids created from aged donors than that of young donors, but failed to attain statistical significance.

Increased Dnmt1 and mtDNA methylation in the cybrids created from aged donors

Global hypo-methylation and local hyper-methylation is known to occur upon aging in the nuclear genome (Terry, Delgado-Cruzata et al. 2011, Jin, Jiang et al. 2014, Reynolds, Taylor et al. 2014, Bacalini, Boattini et al. 2015, Jung and Pfeifer 2015, Yuan, Jiao et al. 2015, Zampieri, Ciccarone et al. 2015). Also, few studies found total DNA methylation to be different in cybrids created from different haplogroups of human mtDNA, thus implying that mtDNA-specific interactions between mitochondria and the nucleus can regulate epigenetic changes (Bellizzi, D'Aquila et al. 2012, Atilano, Malik et al. 2015). Moreover as we have observed differences in mRNA levels of nuclear-encoded genes (**Figure 6D-H**), we wanted to investigate if age-dependent changes in mtDNA affects the global DNA methylation in our cybrids.

To determine the global DNA methylation levels, total cellular DNA was extracted, digested and HPLC-ESI-MS/MS assay was performed. Global DNA methylation did not decrease in the cybrids made from aged donors (**Figure 8A**). Global DNA methylation levels for each cybrid line were further normalized for the total levels of de-oxy-cytidine and the sum of de-oxy-cytidine and 5-methyl- de-oxy-cytidine present in the respective cybrid line's total DNA and were found to be comparable between the cybrids created from young and aged donors (**Figure 8B-C**).

Multiple studies have reported significant alteration of mtDNA methylation in pathologic conditions such as Down's Syndrome (Infantino, Castegna et al. 2011), Amyotrophic lateral sclerosis (Wong, Gertz et al. 2013), non-alcoholic fatty liver disease (Pirola, Gianotti et al. 2013) and also during development in the brain (Ghosh, Sengupta et al. 2014). Limited data is available about age-dependent changes in the mtDNA methylation. While one group has reported that mtDNA methylation does not change (Dzitoyeva, Chen et al. 2012), another group reported that mtDNA methylation changes in an age-dependent manner (Iacobazzi, Castegna et al. 2013). As we found a significant alteration in few of the mtDNA-encoded genes (**Figure 7A, E**) and considering a potential role of mtDNA methylation in regulating mitochondrial transcription (Shock, Thakkar et al. 2011), we investigated mtDNA methylation in our model of aging cybrids by HPLC-ESI-MS/MS assay. Absolute levels of mtDNA methylation did not differ significantly between the cybrids created from young and aged donors but appear to be higher in aged donor group (**Figure 8D**). It should be mentioned that the high intra-group variation in the mtDNA methylation levels in the cybrids lines could have masked any significant difference. Thus, the mtDNA methylation levels for each cybrid line were normalized for the amount of de-oxy-cytidine and also for the sum of de-oxy-cytidine and 5methyl-deoxy-cytidine as internal controls to account for inter-sample variation in digestion. The resulting percentages of mtDNA methylation were found to be significantly elevated (by 40%) in the cybrids generated from aged donors (**Figure 8E-F**).

DNA Methyl transferases (DNMTs) transfer methyl groups to the 5' position of deoxycytidine in DNA and the resulting 5-methyl cytosine are known to regulate gene expression. Presence of mitochondrial isoform of DNMT1 (mtDNMT1) was recently reported, interacting with mtDNA at the CpG sites (Shock, Thakkar et al. 2011). As we found that the percentage of mtDNA

methylation had altered significantly, mRNA levels of mtDNMT1 and total DNMT1 protein levels were examined. We found that relative mRNA levels of mtDNMT1 were significantly increased (2.75 times) in the cybrids generated from the aged donors than the young donors by more than two fold (**Figure 8G**). Subsequently, we found that the cybrids created from aged donors had significantly higher levels of DNMT1 protein (**Figure 8H and I**) in concordance with the increased percentage of mtDNA methylation found in this group.

4.5 DISCUSSION

Alterations in mtDNA have been strongly correlated with age-associated mitochondrial dysfunction. However, there is a lack of evidence that support a direct role of mtDNA in the mitochondrial dysfunction which is known to occur during aging and age-related disorders. In our study, cybrid cell lines created through transfer of platelet-mitochondria from young and old subjects, show altered bioenergetic function. Age-associated mitochondrial dysfunction, in terms of decreased COX holoenzyme function and basal-mitochondrial oxygen consumption were found in the cybrids created from aged donors. Thus, we have demonstrated a direct role for human mtDNA in causing mitochondrial dysfunction during aging. Additionally, increase in mRNA levels of some of the mtDNA-encoded and nuclear-encoded genes that promote mitochondrial biogenesis, mRNA levels of mitochondrial isoform of DNMT1, total protein levels of DNMT1 and mtDNA methylation were observed in the cybrids made from aged donors.

Majority of the aging studies have only established correlations between mtDNA alterations and mitochondrial dysfunction using skin fibroblasts, organs of animal models and clinical samples. Limited studies have addressed the direct role of mtDNA in aging. Our results regarding direct role of age-associated alterations in the mtDNA in causing mitochondrial dysfunction are

supported by previous mouse cybrid studies, which have used mitochondria from skeleton muscle and synaptosomes from mice of different ages (Li, Li et al. 2010, Li, Kumar Sharma et al. 2013). Very few studies have examined the direct role of mtDNA from human subjects in age-dependent mitochondrial dysfunction, and have arrived at contradictory conclusions (Hayashi, Ohta et al. 1994, Laderman, Penny et al. 1996, Isobe, Ito et al. 1998). All these studies have used skin fibroblast as the source of mtDNA, but used different approaches (nuclear hybrid or cytoplasmic hybrids) or used different nuclear background (i.e. different ρ^0 cells). Study outcomes in cybrid models are influenced by the nuclear background of the ρ^0 cell line that has been used to generate the cybrid cell lines (Khan, Smigrodzki et al. 2007, Wilkins, Carl et al. 2014). Even if mitochondria used to generate the cybrid cell lines are obtained from a common source, they might not function similarly in the context of different nuclear backgrounds. Thus, variations in cybrid technology coupled with difference in approaches might have resulted in conflicting findings. All these studies have based their conclusions by using the clones obtained after generating the transformed cybrid cell line (Hayashi, Ohta et al. 1994, Laderman, Penny et al. 1996). On one hand, Laderman *et al.* found that only 5% of the clones obtained from the old-transformants were respiration-deficient (Laderman, Penny et al. 1996) whereas Hayashi *et al.* and Isobe *et al.* worked with clones of a very small group of individuals (Hayashi, Ohta et al. 1994, Isobe, Ito et al. 1998). Our studies were conducted in the transformed cybrid cell line itself, which not only is more analogous to the heteroplasmy that is observed in physiology, but also with a larger sample set of transformed cell lines generated from 9 young and 7 aged human donors making our study more relevant. Moreover, we have explored bioenergetic fluxes and associated parameters in more detailed manner compared to previous human studies (Hayashi, Ohta et al. 1994, Laderman, Penny et al. 1996, Isobe, Ito et al. 1998) and shown for the first time

that age-dependent alterations of human mtDNA determines reduced COX activity during aging, which is consistent with various correlative studies in animal and bio-specimens. Further our data suggest that decreased COX activity may be responsible for reduced mitochondrial oxygen consumption observed in the cybrids generated from aged donors.

In our model of aging using cybrids, we did not observe age-dependent alteration of energy (ATP levels, ADP/ATP), redox states (NAD^+/NADH), glycolysis and decrease in Complex I activity, as observed in some other aging studies. In our model, cybrids were created with a common nuclear background of the SH-SY5Y p0 cells and the only variable factor was the platelet-mitochondria that were obtained from different aged subjects. This suggests the possibility of predominant control of the nuclear genome over these bioenergetic parameters, which failed to alter in an age-dependent manner. Other studies indicating regulation of mitochondrial dysfunction by nuclear genome during aging support such a possibility (Hayashi, Ohta et al. 1994, Isobe, Ito et al. 1998). Paradoxically, we observed an increase in Complex I activity in the cybrids generated from aged donors, suggesting a compensatory response to decreased mitochondrial OCR and COX activity. This may be another reason why some of the bioenergetic parameters remained unaltered. Overall, induction of mRNA levels of nuclear-encoded genes that promote mitochondrial biogenesis (PRC, NRF1, NRF2 and TFAM) and some of the mtDNA-encoded OXPHOS genes (MT-ND1 and MT-ND5), further support a potential compensatory phenomenon in the cybrids created by transfer of platelets from aged human donors.

Similar to Laderman *et.al* (Laderman, Penny et al. 1996), we have found that age-dependent changes in mtDNA do not have an effect on mtDNA copy number, suggesting possibility of nuclear dominance over controlling mtDNA copy number during aging. However, it needs to be

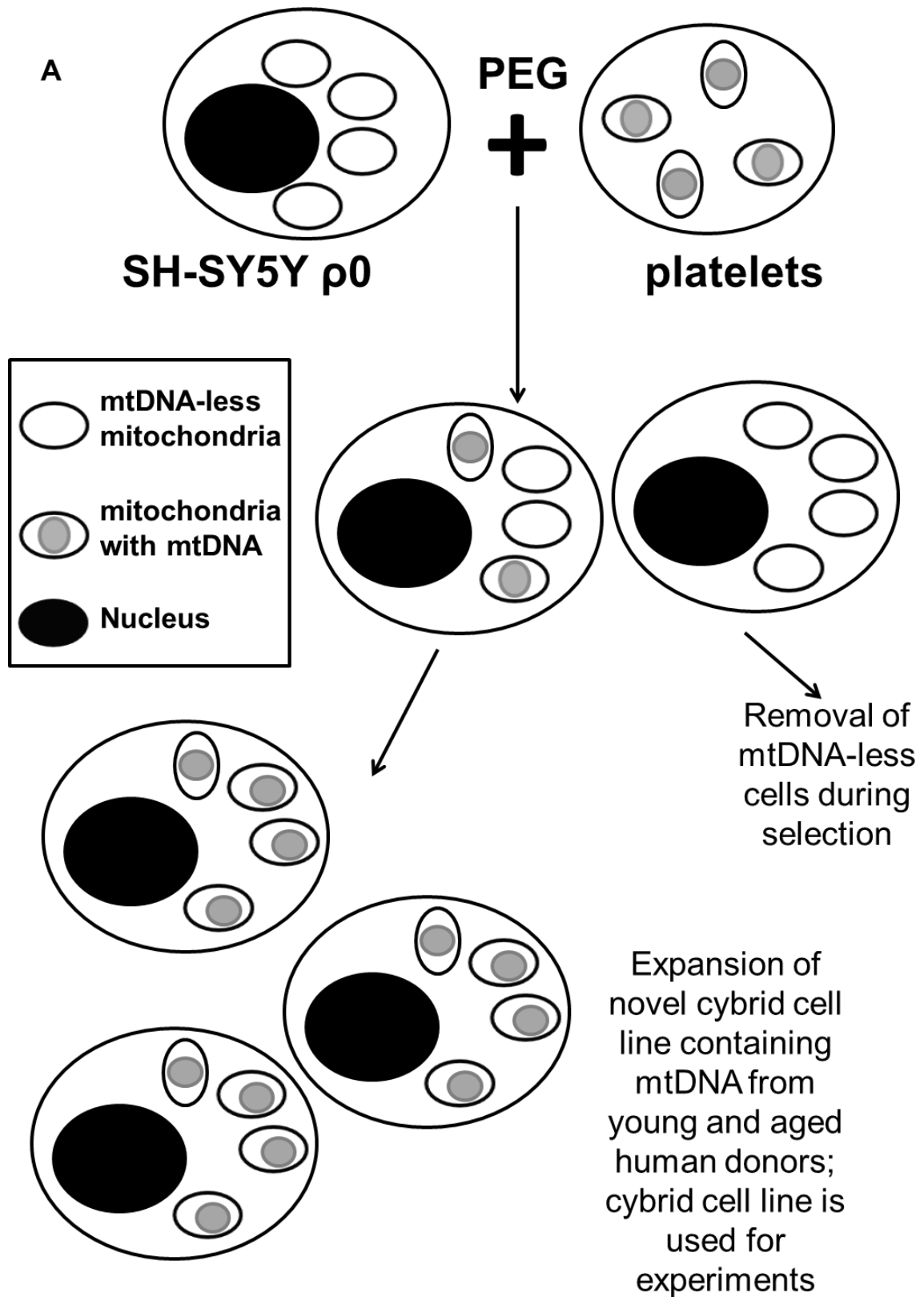
mentioned that inconsistency exists with regards to age-dependency of the mtDNA copy number in the literature; while some reports suggested that there is no alteration in mtDNA copy number (Laderman, Penny et al. 1996, Miller, Rosenfeldt et al. 2003, Frahm, Mohamed et al. 2005, Mohamed, Hanke et al. 2006), others reported it to be decreased (Welle, Bhatt et al. 2003, Short, Bigelow et al. 2005, Cree, Patel et al. 2008) or even increased (Lee, Pang et al. 1994, Barrientos, Casademont et al. 1997, Pesce, Cormio et al. 2001) upon aging. Considering that mtDNA copy number is a tissue-specific characteristic, even reports about mtDNA copy number from a single tissue such as skeletal muscle are inconsistent. Few studies have reported mtDNA copy number in the aging muscle to be unaltered (Miller, Rosenfeldt et al. 2003, Frahm, Mohamed et al. 2005) whereas others concluded an increase (Barrientos, Casademont et al. 1997, Pesce, Cormio et al. 2001) or decrease (Welle, Bhatt et al. 2003, Short, Bigelow et al. 2005) in mtDNA copy number. Irrespective of these inconsistencies, our studies indicate that age-dependent changes in mtDNA are not a determining factor for controlling mtDNA copy number.

DNA methylation can regulate gene-expression and also known to change in an age-dependent manner (Terry, Delgado-Cruzata et al. 2011, Jin, Jiang et al. 2014, Reynolds, Taylor et al. 2014, Bacalini, Boattini et al. 2015, Jung and Pfeifer 2015, Yuan, Jiao et al. 2015, Zampieri, Ciccarone et al. 2015). As significant alteration in mRNA levels of nuclear-encoded genes that promote mitochondrial biogenesis and some of the mtDNA-encoded genes were observed, we explored total DNA methylation and mtDNA methylation in the cybrids created from young and aged donors. We did not find a significant difference in total DNA methylation in our cybrid model; suggesting that age-dependent changes in mtDNA does not have a role in global DNA methylation. However, this does not negate the possibility of gene-specific changes in methylation in the nuclear genome that might occur during age-associated mitochondrial

dysfunction and that they can be regulated by mtDNA alterations, which have not been pursued in this study. Limited data is available regarding mtDNA methylation or its alteration during aging. Our study, for the first time reported that total levels of DNMT1 protein, relative mRNA levels of mitochondrial isoform of DNMT1 and percentage methylation in the mtDNA were significantly elevated in the cybrids created from aged donors. As HPLC-ESI-MS/MS assay used in our study cannot differentiate nucleosides obtained from mtDNA or nuclear DNA and even though the mtDNA has been extracted from isolated mitochondria, chances of nuclear DNA contamination cannot be excluded completely. However, we have observed that the mtDNA prepared in this manner is enriched by 50 fold for mtDNA with respect to total DNA and 99.71 % of the nuclear DNA is eliminated. Moreover, our data indicated that the total DNA methylation did not change (or trended to be decreased) whereas the mtDNA methylation was increased in cybrids from aged donors, suggesting that confounding presence of nuclear DNA contamination would not affect interpretation of our results that mtDNA methylation is increased in cybrids from aged donors. Our results suggest the possibility that age-dependent alterations in mtDNA may play a role in determining DNA methylation-associated parameters. An alternative explanation could be that the age-dependent differences in mtDNA methylation existed in the source mtDNA itself (i.e. the platelet-mtDNA), even before the cybrids were made. However, irrespective of both the scenarios, concomitant age-dependent variation in expression levels of genes that promote mitochondrial biogenesis, DNMT1 protein and mtDNA methylation illustrates the possibility that mtDNA methylation, DNMT1 and cellular-bioenergetics could be related in an age-dependent manner, but the mechanism behind remains unclear and needs to be studied in details. Thus, our results support the rationale to explore the field of mitochondrial-epigenetics.

In conclusion, using cybrid cell lines created through transfer of platelet-mitochondria from young and aged human subjects, we demonstrated that age-dependent changes in mtDNA have a direct role in determining bioenergetic flux, electron transport chain function and expression of genes that control mitochondrial biogenesis. Further, we found that mtDNA methylation is altered in age-dependent manner in cybrid model. Furthermore, our study indicates that age-dependent alterations in mtDNA may not be the sole determinant of mitochondrial dysfunction observed during aging, but simultaneous effects of dysregulation via nuclear genome may have a role to play in the age-associated mitochondrial dysfunction.

4.6 FIGURES



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B Cybrids generated
from young
mitochondria
donors (YDC)

Sex	Age	Cell line ID
F	29	YDC-1
M	30	YDC-2
F	32	YDC-4
F	33	YDC-5
F	27	YDC-6
F	24	YDC-7
F	31	YDC-8
M	28	YDC-9
M	28	YDC-10

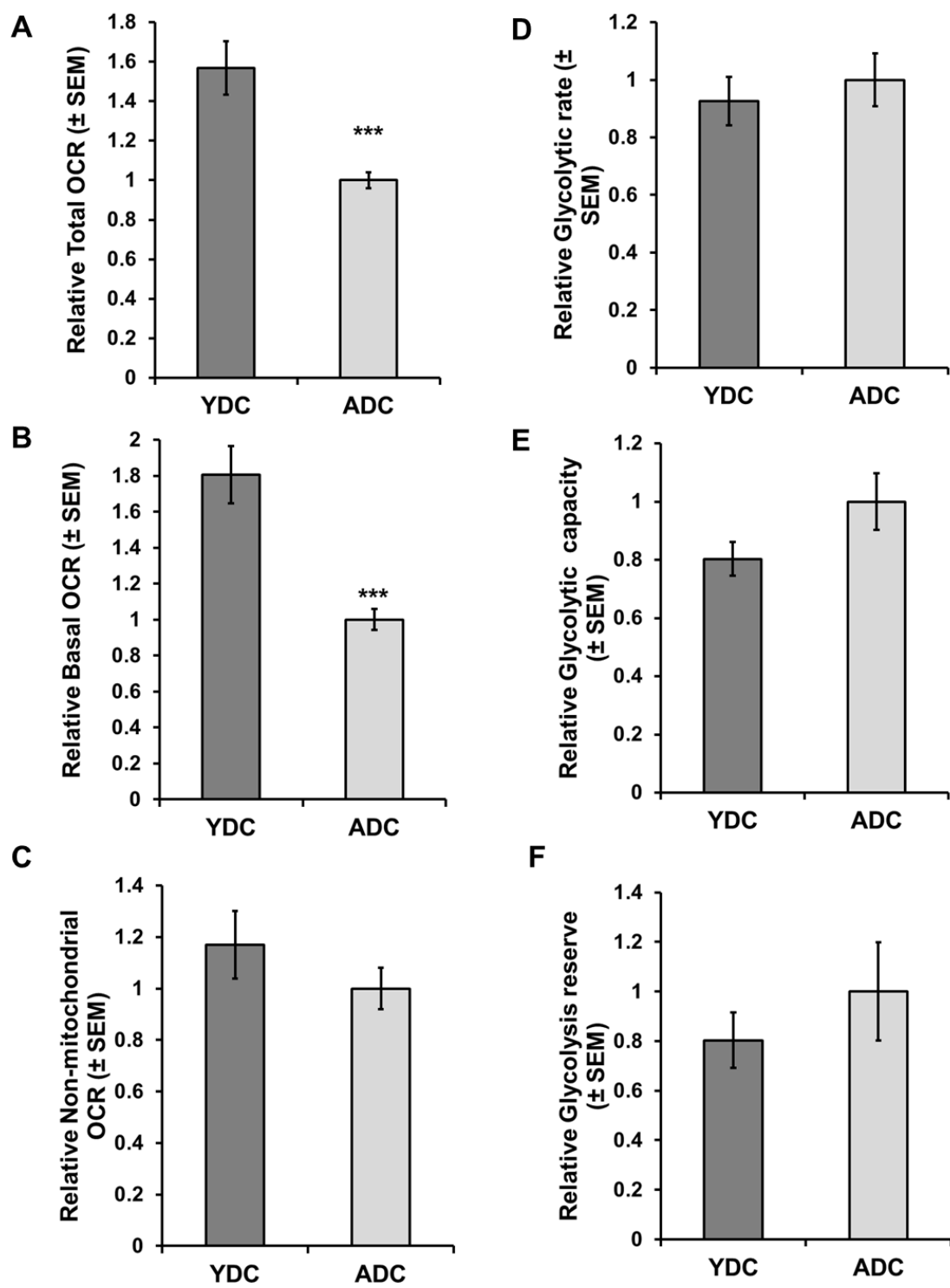
Average age	29.111111
sem	0.9196081

Cybrids generated
from aged
mitochondria
donors (ADC)

Sex	Age	Cell line ID
M	68	ADC-2
M	85	ADC-3
F	85	ADC-4
M	65	ADC-5
M	71	ADC-6
M	79	ADC-9
F	73	ADC-10

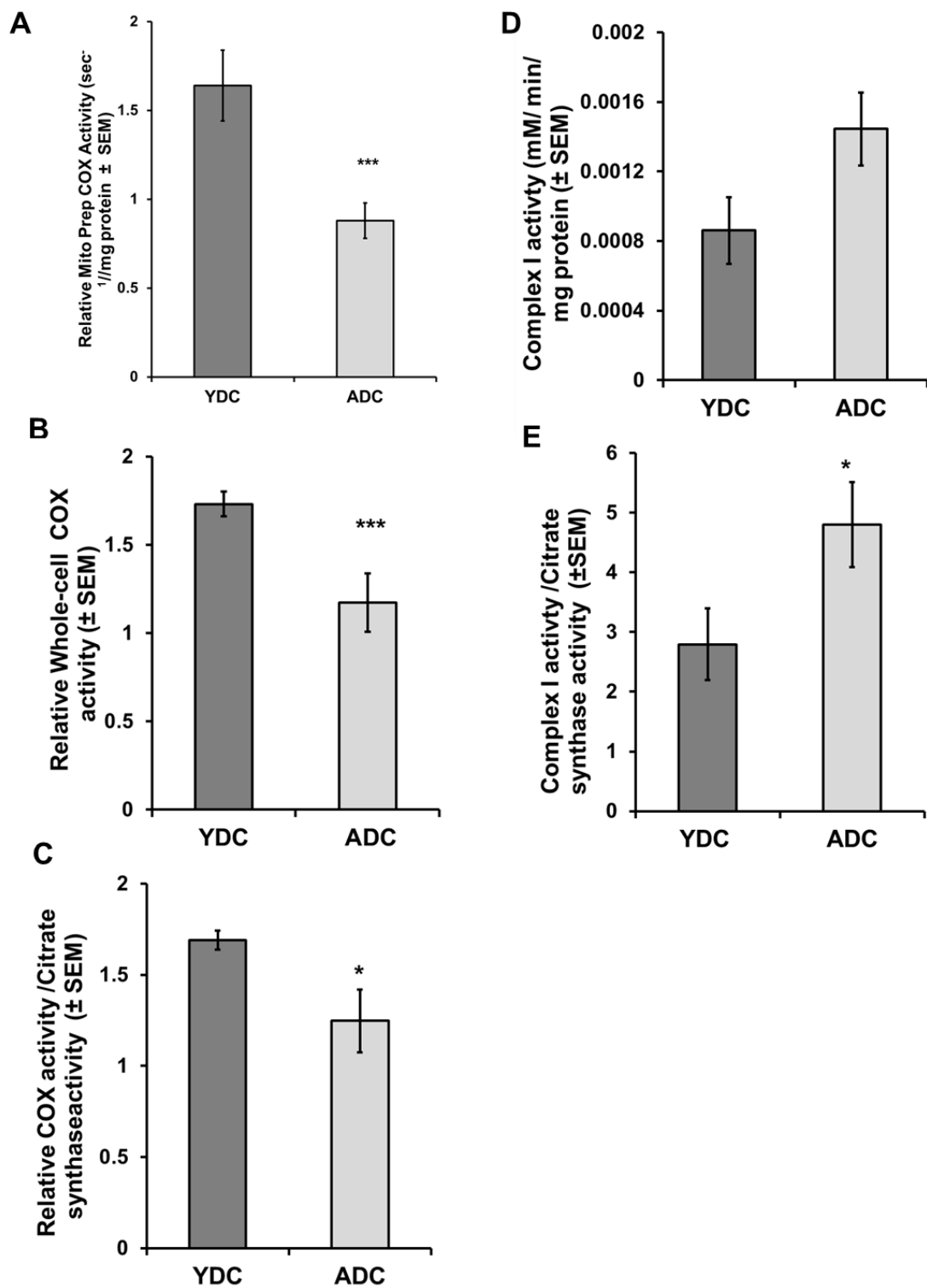
Average age	75.142857
sem	3.0270886

Figure 1. Schematic representation of preparation of human cybrids: (A) Platelets isolated from blood of 9 young donors (mean age=29.1 \pm 0.9 years) and 7 aged donors (mean age=75.1 \pm 3.01 years) were fused with mtDNA depleted SH-SY5Y ρ^0 cells by Poly-Ethylene-Glycol (PEG). After a week of expansion, mtDNA-less cells were removed by culturing them in selection medium for 6 weeks thus resulting in 9 and 7 cybrid lines generated from young and aged human donors respectively, that have a common nuclear background but different mitochondrial DNA. (B) Tabular representation of the sex and age for the young and aged human donors.



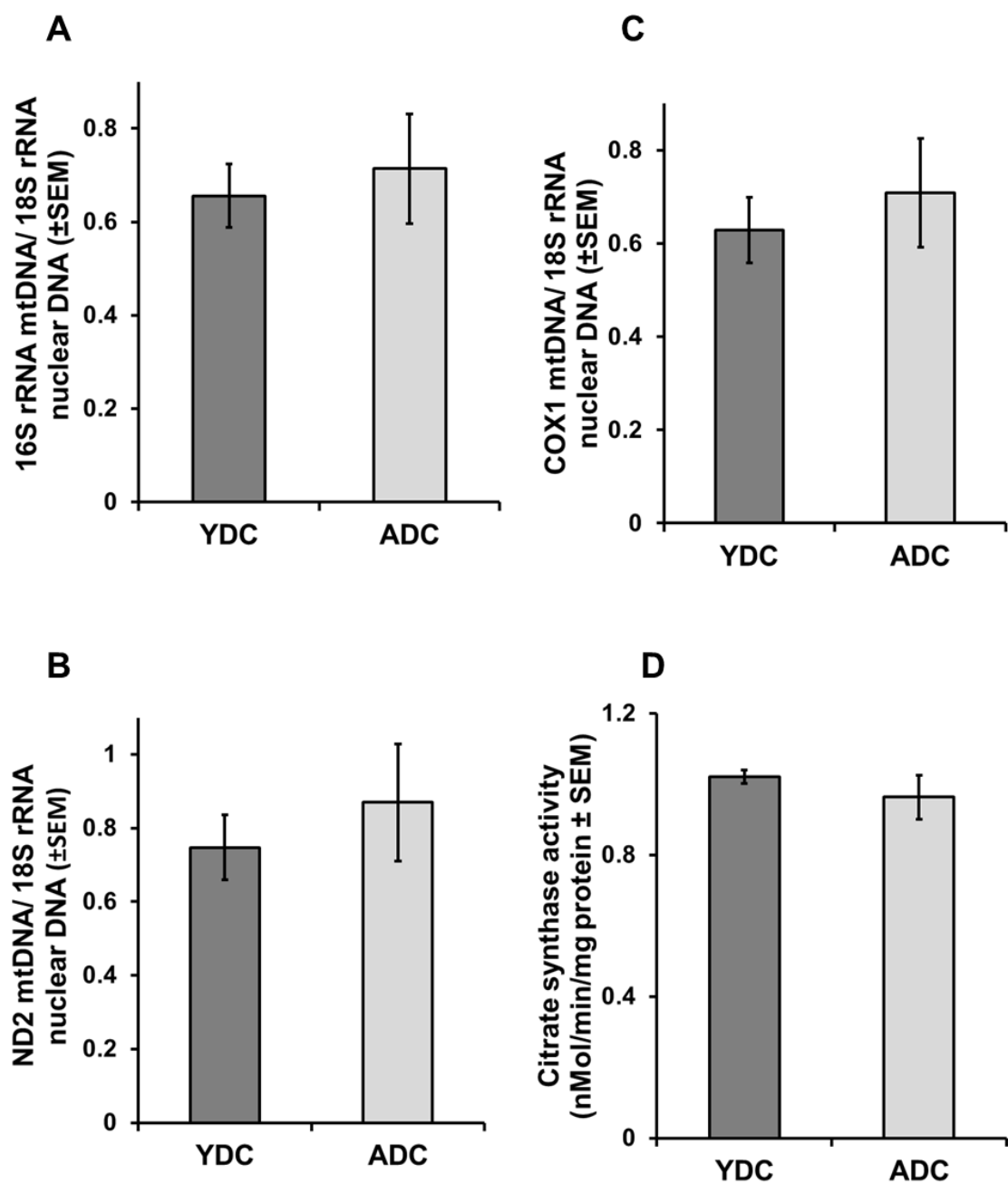
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Figure 2. Oxygen Consumption Rate (OCR) and Extra-Cellular Acidification Rate (ECAR) parameters in the cybrids created from young and aged donors: (A) Total OCR, (B) Basal-Mitochondrial OCR, (C) Non-Mitochondrial OCR, (D) Glycolysis rate, (E) Glycolysis capacity and (F) Glycolysis reserve were determined for each cybrid line, compared between the two groups and then shown. * indicates a significant difference between groups at $p < 0.05$; ** indicates a significant difference between groups at $p < 0.01$.



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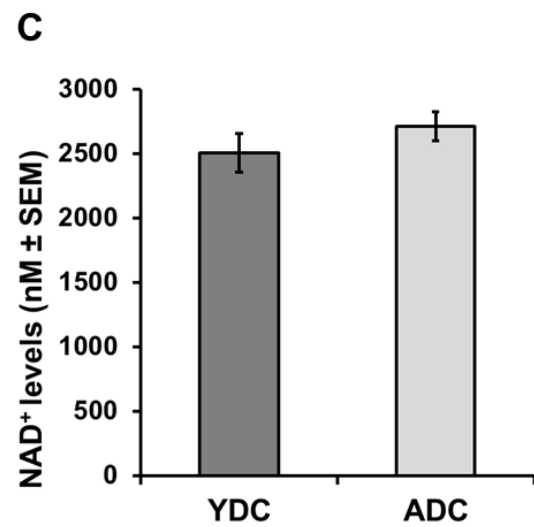
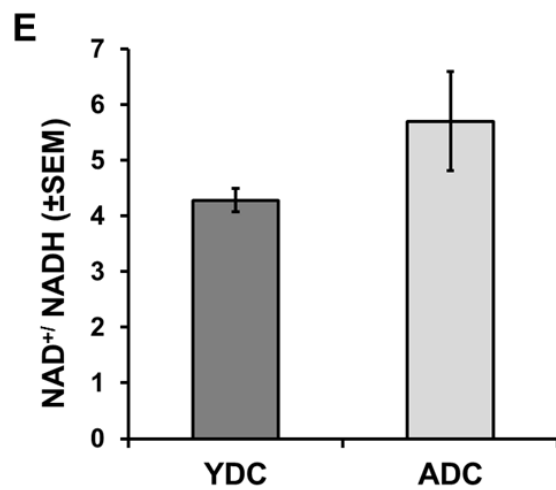
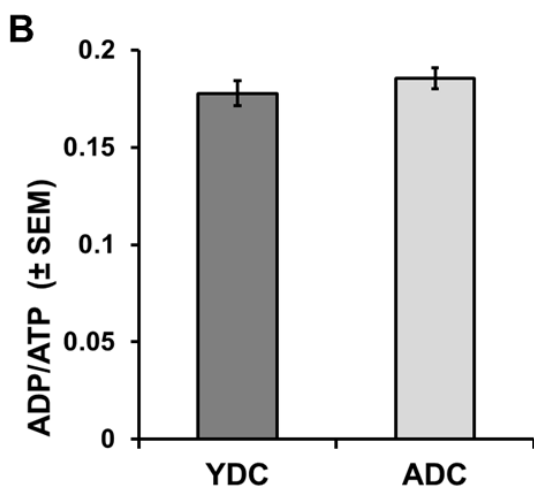
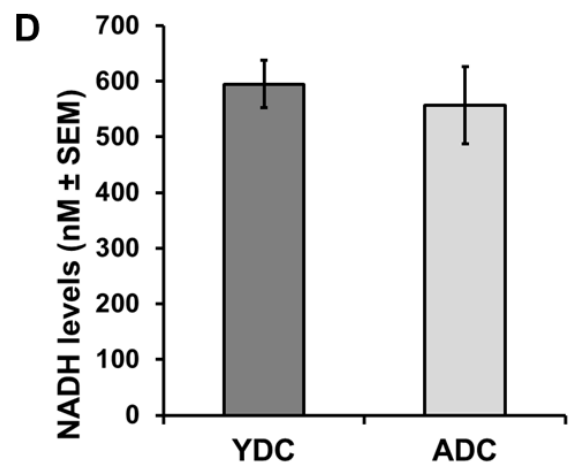
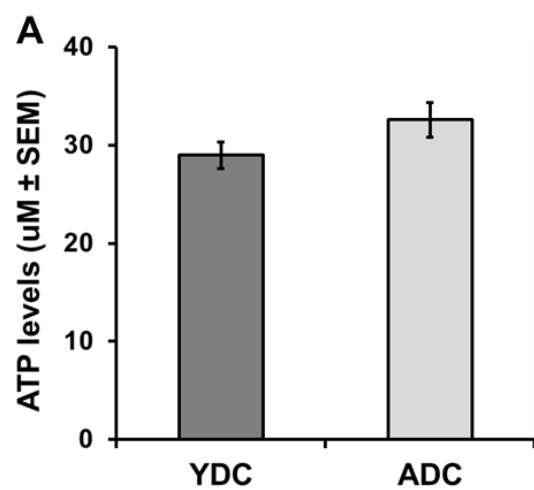
Figure 3. Cytochrome c oxidase (COX) assay and Complex I assay in the cybrids created from young and aged donors: For COX assay, isolated mitochondria and whole-cell were used to determine the rate constant (V_{\max}) spectrophotometrically by following the conversion of reduced cytochrome c to oxidized cytochrome c. Each cybrid line's rate constant was normalized to the amount of protein in (A) the mitochondrial preparation or (B) lysed whole cell in its assay cuvette to provide a rate in $\text{sec}^{-1}/\text{mg}$ protein. Citrate synthase assay was performed for each cybrid line and was used to normalize the respective COX V_{\max} obtained in (B) and then compared in (C) Relative COX activity/Citrate synthase activity. For Complex I assay, the rate constant (V_{\max}) was spectrophotometrically determined by following the oxidation of NADH to NAD^+ . Each cybrids line's rate was then normalized to the amount of protein in the mitochondrial preparation and divided by the extinction coefficient of the dye in both the groups to provide (D) Complex I activity. The complex I activity was then further normalized by the citrate synthase to provide (E) Complex I activity/Citrate Synthase activity for both the group of cybrids. * indicates a significant difference between groups at $p < 0.05$; ** indicates a significant difference between groups at $p < 0.01$; *** indicates a significant difference between groups at $p < 0.001$.



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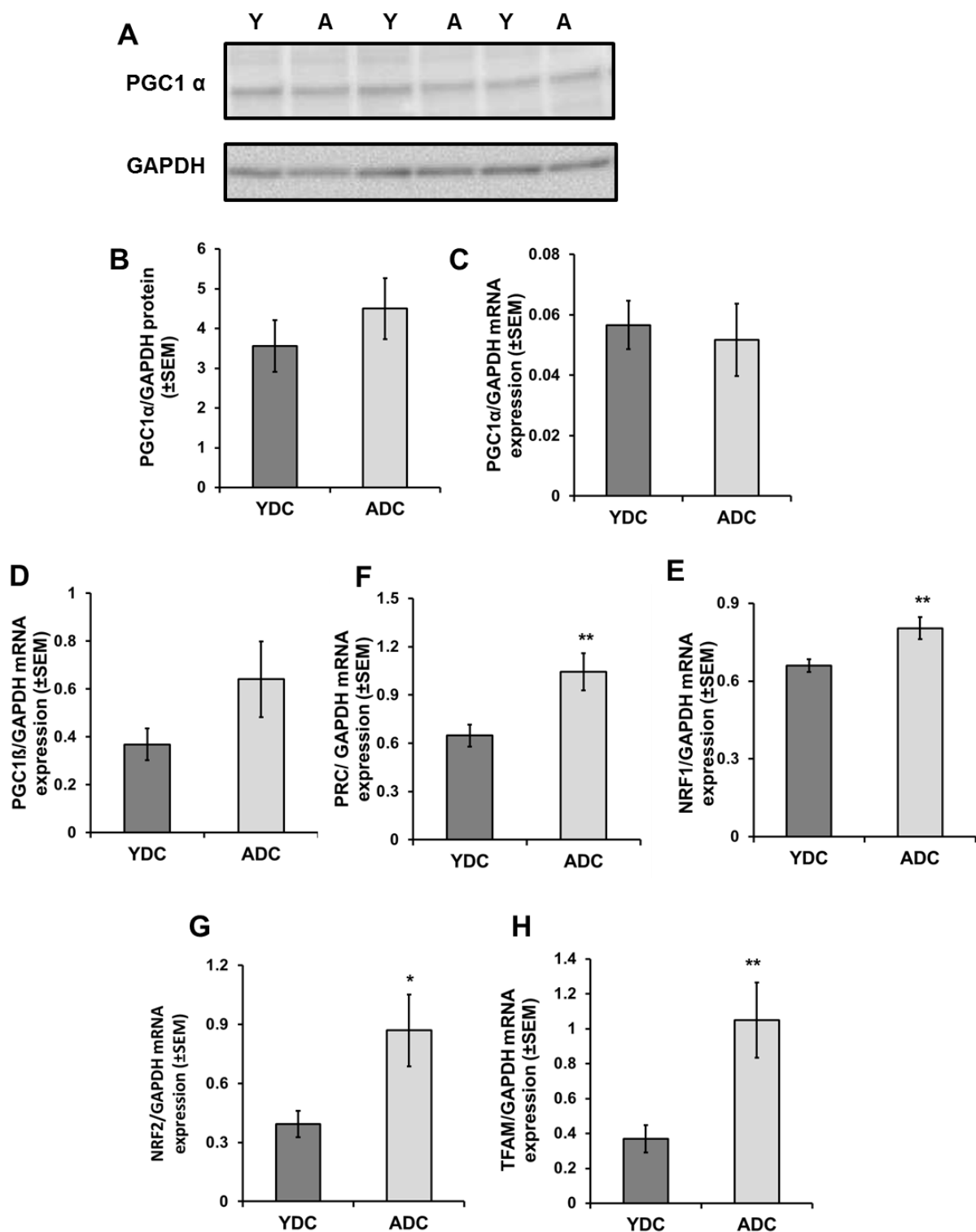
Figure 4. Mitochondrial mass markers in the cybrids created from young and aged donors:

MtDNA levels relative to the nuclear DNA (18S rRNA) was estimated for each cybrid line by using primers to amplify (A) 16S rRNA, (B) MT-ND2 and (C) MT-COX1 from total DNA in both the groups of cybrids. For Citrate synthase assay, linear formation of 5-mercapto-2-nitrobenzoic acid is followed spectrophotometrically at 412 nm to provide the rate, whose extinction coefficient is $0.0136 \mu\text{M}^{-1}\text{cm}^{-1}$. The rate was then normalized for the amount of protein in the lysed whole cells to provide (D) Citrate synthase activity.



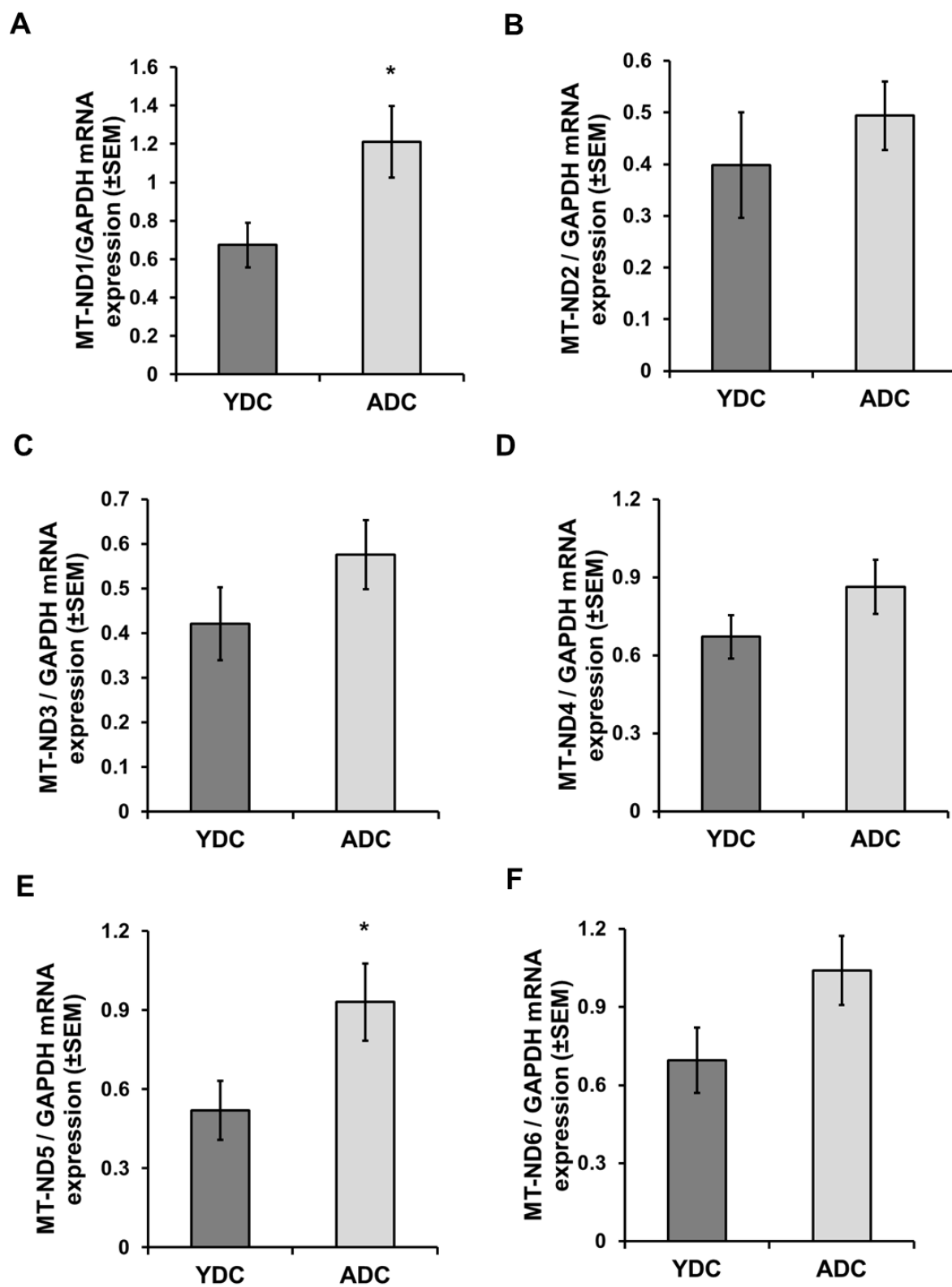
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Figure 5. Energy and redox intermediates in the cybrids created from young and aged donors : (A) ATP levels (μM), (B) ADP/ATP ratio, (C) NAD^+ levels (nM), (D) NADH levels (nM) and (E) NAD^+/NADH ratios were determined for each cybrid line in both the groups.



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Figure 6. Transcriptional co-activators and transcription factors that promote mitochondrial biogenesis in the cybrids created from young and aged donors: Relative levels of PGC1 α protein (A and B) and (C) mRNA are shown. Relative mRNA levels of (D) PGC1 β , (E) PRC, (F) NRF1, (G) NRF2 and (H) TFAM are shown. * indicates a significant difference between groups at $p < 0.05$; ** indicates a significant difference between groups at $p < 0.01$.



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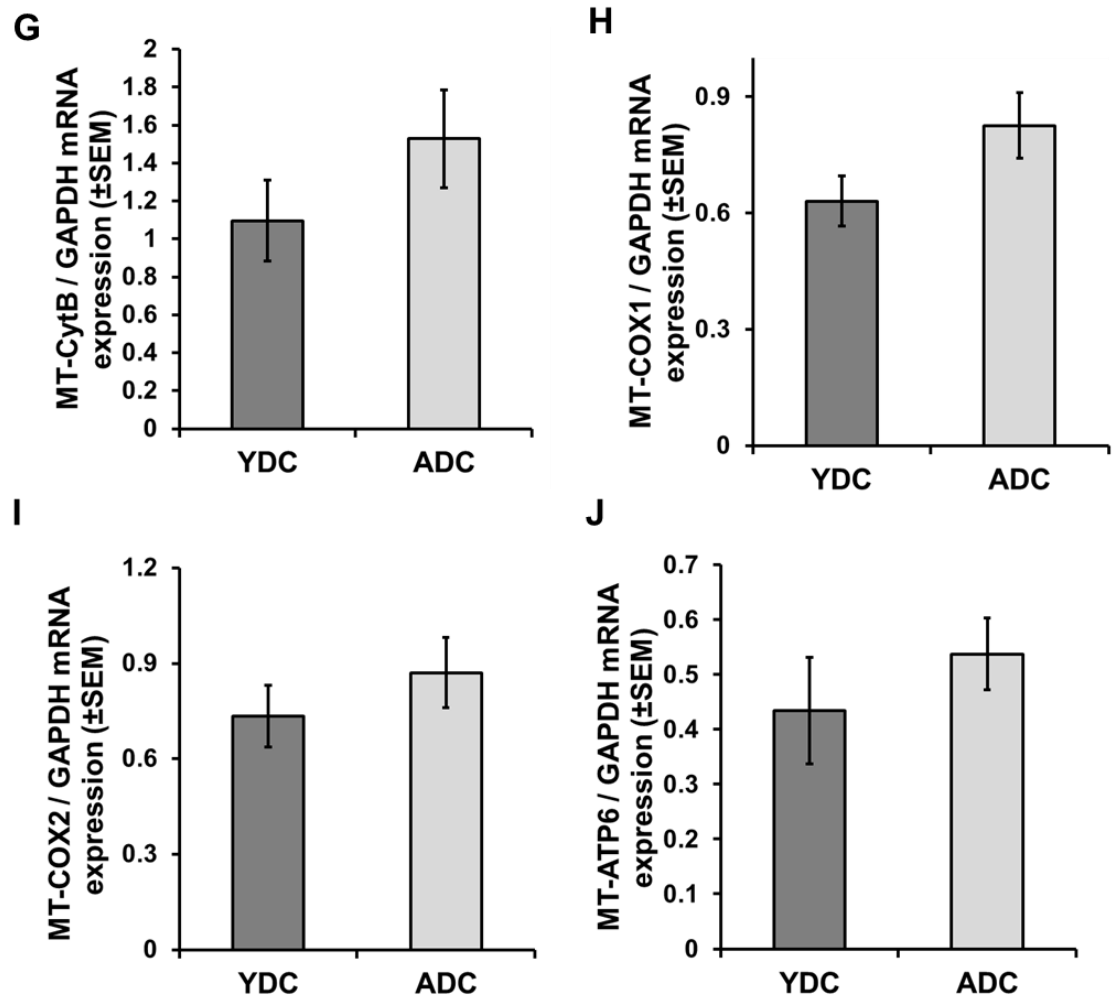
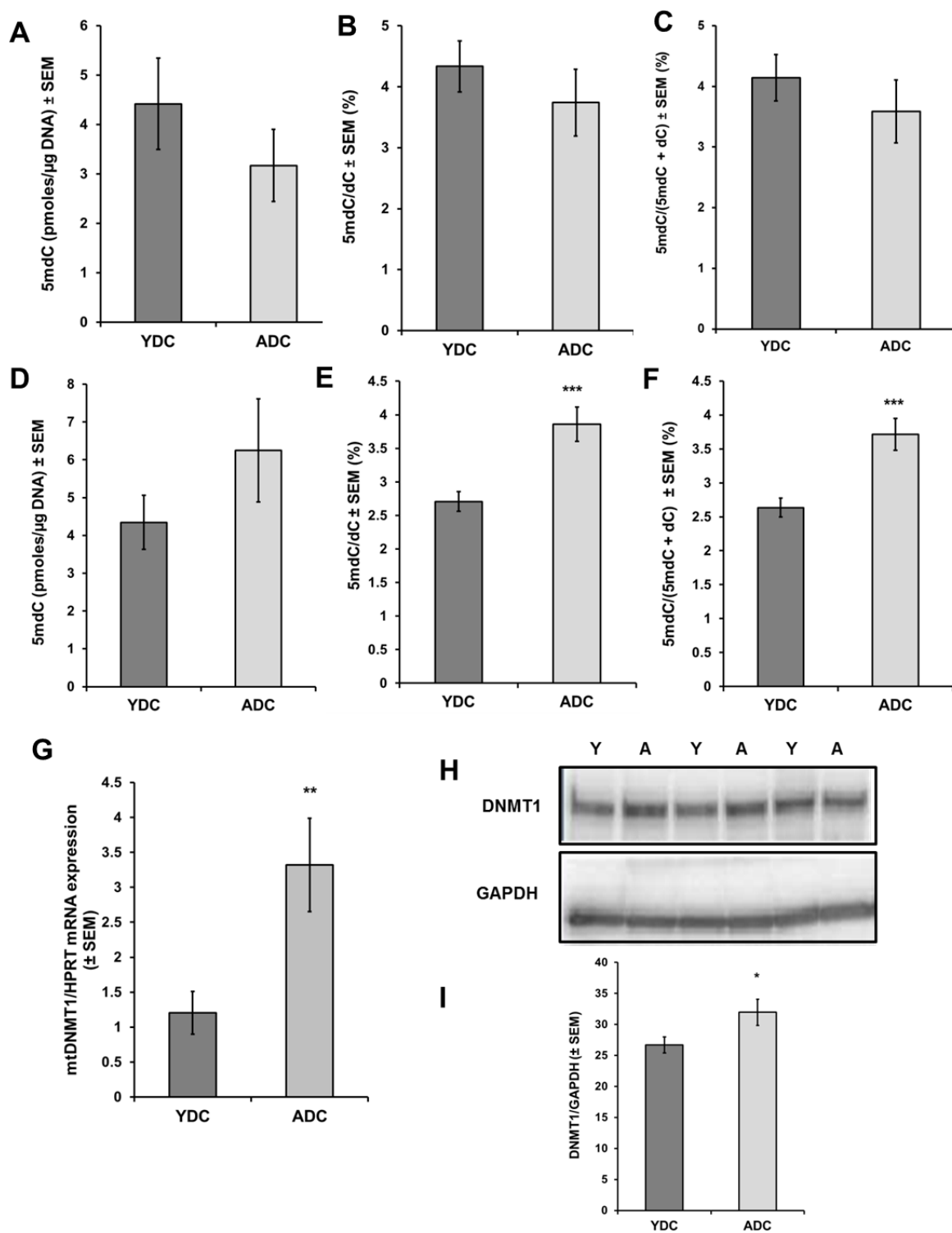


Figure 7. Relative mRNA expression of OXPHOS genes in the cybrids created from young and aged donors: Relative mRNA levels of (A) MT-ND1, (B) MT-ND2, (C) MT-ND3, (D) MT-ND4, (E) MT-ND5, (F) MT-ND6, (G) MT-COX1, (H) MT-COX2, (I) MT-CytB and (J) MT-ATP6 are shown. * indicates a significant difference between groups at $p < 0.05$.



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Figure 8. Global DNA methylation, mtDNA methylation and DNMT1 expression in the cybrids created from young and aged donors : Global DNA methylation was estimated from the total DNA for each cybrid line in pmoles/ μ g DNA (A) and normalized by the amount of dC and the sum of dC and 5mdC in every cybrid line's total DNA and represented as percentage in (B) and (C) respectively. Mitochondrial DNA methylation was estimated in the mitochondrial DNA for each cybrid line in pmoles/ μ g mtDNA as represented in (D) and normalized by the amount of dC in (E) and also the sum of dC and 5mdC in (F) for every cybrid line's mitochondrial DNA and represented as percentages. Relative mRNA levels of mtDNMT1 is compared and shown in (G). Relative levels of DNMT1 protein in whole cell lysates are shown in (H and I). * indicates a significant difference between groups at $p < 0.05$; ** indicates a significant difference between groups at $p < 0.01$; *** indicates a significant difference between groups at $p < 0.001$.

CHAPTER V: DISCUSSION AND CONCLUSION

5.1 STATE OF THE FIELD AT THE ONSET OF THIS DISSERTATION WORK AND ITS PROGRESS

MtDNA methylation has remained ignored for four decades until the recent resurgence in the field. When this dissertation work was proposed, mtDNA methylation was controversial with only eight studies that claimed it to be present (Vanyushin, Kiryanov et al. 1971, Nass 1973, Vanyushin and Kirnos 1977, Shmookler Reis and Goldstein 1983, Pollack, Kasir et al. 1984, Chestnut, Chang et al. 2011, Infantino, Castegna et al. 2011, Shock, Thakkar et al. 2011), whereas three other studies asserted its absence (Dawid 1974, Groot and Kroon 1979, Maekawa, Taniguchi et al. 2004). As most of these studies had used methods with poor sensitivity for detecting mtDNA methylation, reproducibility issues and possibilities of artifacts could not be refuted. After discovery of mitochondrial isoform of DNMT1 (mtDNMT1) in 2011 (Shock, Thakkar et al. 2011), there was resurgence in the field with several studies reporting presence of mtDNA methylation in different model systems (Dzitoyeva, Chen et al. 2012, Kelly, Mahmud et al. 2012, Bellizzi, D'Aquila et al. 2013, Byun, Panni et al. 2013, Jia, Li et al. 2013, Pirola, Gianotti et al. 2013, Wong, Gertz et al. 2013, Ghosh, Sengupta et al. 2014, Janssen, Byun et al. 2014, Baccarelli and Byun 2015, Ferreira, Serafim et al. 2015, Janssen, Byun et al. 2015, Zheng, Linarelli et al. 2015) and a single study reported its absence (Hong, Okitsu et al. 2013). However, debate revolving presence of mtDNA methylation still has not ended, as concerns with regards to the limitations of utilized methods have been raised (van der Wijst and Rots 2015). Moreover, the likely prospects for presence of nuclear DNA contamination in the preparation of mtDNA have not been so far critically addressed and most of the studies have not used purified mtDNA for determining mtDNA methylation.

At the onset of this dissertation work, only two studies had reported changes in mtDNA methylation in pathological conditions using samples from patients inflicted with Down's syndrome and ALS (Chestnut, Chang et al. 2011, Infantino, Castegna et al. 2011). With the renewed interest in the field after discovery of mtDNMT1, multiple other studies reported changes in mtDNA methylation in bio-specimens obtained from several physiological states and pathophysiological conditions (Dzitoyeva, Chen et al. 2012, Feng, Xiong et al. 2012, Kelly, Mahmud et al. 2012, Byun, Panni et al. 2013, Jia, Li et al. 2013, Pirola, Gianotti et al. 2013, Wong, Gertz et al. 2013, Ghosh, Sengupta et al. 2014, Janssen, Byun et al. 2014, Baccarelli and Byun 2015, Ferreira, Serafim et al. 2015, Janssen, Byun et al. 2015, Zheng, Linarelli et al. 2015). A few of these studies have also reported concomitant changes in expression levels of some of the mtDNA-encoded genes (Shock, Thakkar et al. 2011, Feng, Xiong et al. 2012, Pirola, Gianotti et al. 2013). However, any role of mtDNA methylation in controlling gene expression of mtDNA-encoded genes and mitochondrial function still remains completely unexplored. Further, how mtDNA gets methylated and whether DNMTs can methylate mtDNA is not yet known. Besides, the field of mtDNA methylation is still at such an incipient state that it has not yet been tested whether mtDNA methylation can be altered experimentally in the first place and whether any such alterations in mtDNA methylation have an association with expression of mtDNA-encoded genes and mitochondrial function.

Nuclear genome is known to undergo global hypo-methylation and gene-specific hyper-methylation during aging (Terry, Delgado-Cruzata et al. 2011, Jin, Jiang et al. 2014, Reynolds, Taylor et al. 2014, Bacalini, Boattini et al. 2015, Jung and Pfeifer 2015, Yuan, Jiao et al. 2015, Zampieri, Ciccarone et al. 2015). Unlike nuclear DNA, whether mtDNA methylation is altered upon aging is not known. Mitochondrial dysfunction is known to occur during aging (Boffoli,

Scacco et al. 1994, Fujita, Yamauchi et al. 1996, Ojaimi, Masters et al. 1999, Short, Bigelow et al. 2005) and positive correlations have been drawn between alterations in mtDNA and the mitochondrial dysfunction that occurs during aging (Vermulst, Wanagat et al. 2008) . However, very few studies have investigated the direct role of aging-associated alterations of mtDNA in causing mitochondrial dysfunction and have arrived at contradictory conclusions (Hayashi, Ohta et al. 1994, Laderman, Penny et al. 1996, Isobe, Ito et al. 1998). Thus, a direct role of mtDNA in aging-associated mitochondrial dysfunction is not yet established and neither it is clear if mtDNA methylation is altered during aging.

5.2 SUMMARY OF FINDINGS

5.2.1 SUMMARY OF FINDINGS: CHAPTER II – DETECTION OF METHYLATION IN MITOCHONDRIAL DNA USING HPLC-MS/MS IN SH-SY5Y NEUROBLASTOMA CELL LINE

To quantitate global levels of 5-mC in SH-SY5Y mtDNA accurately, a highly specific and direct assay was established using HPLC-ESI-MS/MS. It is known that differential or incomplete DNA digestion is the pitfall for quantifying 5-mC using HPLC-ESI-MS/MS that can lead to inaccurate quantification of 5-mC (van der Wijst and Rots 2015). This limitation was not addressed by the single study that has studied mtDNA methylation using HPLC-MS/MS (Infantino, Castegna et al. 2011). In our study, a single-step method for digesting mtDNA was used to minimize variabilities in digestion. Also, a novel approach of utilizing oligonucleotides containing known amounts of 5-mC and cytosine as reference standards was adopted for quantification of 5mC to account for differential digestion. Another limitation for studying mtDNA methylation is the chances of nuclear DNA contamination, which was addressed by determining 5-mC in gradient

purified SH-SY5Y mitochondria. Additionally, the purity of our mtDNA preparations was characterized at both protein and DNA levels.

Initial mass-spectrophotometric characterization of the nucleosides using mixture of analytical standards was carried out where each nucleoside resolved distinctly and was detected in a linear manner. Further, linear and proportional detection of all nucleosides in the digestion products of our reference standards (oligonucleotides containing known amounts of 5-mC and Cytosine) was observed. No 5-mC was detected in the digestion mixture of oligonucleotides that did not contain the nucleoside 5-mC, thus indicating that our assay is specifically detecting 5-mC.

Next, the range of linear and proportional digestion for SH-SY5Y mtDNA was established. 5-mC levels were quantitated and compared between whole-cell DNA, crude and gradient-purified mtDNA and were found to be comparable (~3% methylation) in all these preparations, which is consistent with the literature. Further, purity characterization at DNA level suggested that more than 99% of nuclear DNA contamination was removed in mtDNA prepared from both crude and gradient-purified mitochondria. Further, linear regression analysis between estimated percentage of mtDNA in various mitochondrial preparations and the corresponding percentage methylation revealed that mtDNA methylation occurs at levels at least as much as that of the nucleus. MtDNA methylation was further confirmed at a specific site in 12S rRNA using methylation-specific PCR.

5.2.2 SUMMARY OF FINDINGS: CHAPTER III – ALTERATIONS IN MTDNA METHYLATION BY DNMT1 OVEREXPRESSION AND 5-AZA-DC TREATMENT AND ITS ASSOCIATION WITH MITOCHONDRIAL TRANSCRIPTION AND MITOCHONDRIAL FUNCTION IN SH-SY5Y NEUROBLASTOMA CELL LINE

To investigate whether mtDNA methylation can be altered and if there are any associated changes in mtDNA-encoded gene expression and mitochondrial function, two distinct experimental approaches were utilized. DNMT1 was overexpressed in SH-SY5Y cells or cells were treated with DNA methylation inhibitor (Decitabine (5-aza-dC)). DNMT1 is previously known to localize in mitochondria (Chestnut, Chang et al. 2011, Shock, Thakkar et al. 2011, Bellizzi, D'Aquila et al. 2013) but whether it can cause mtDNA methylation is not yet known. Further, whether DNA methylation inhibitor can alter mtDNA methylation was not explored previously. Overexpression of DNMT1 increased global mtDNA methylation by 2.5 fold whereas 5-aza-dC treatment decreased it by half in SH-SY5Y mtDNA as studied using HPLC-ESI-MS/MS. Similar results were obtained when methylation-specific PCR was used to study mtDNA methylation at a specific site in 12S rRNA. Thus, our data suggested that mtDNA methylation is an alterable phenomenon which can be affected by DNMT1 levels and DNA methylation inhibitor treatment. As we found that overexpression of DNMT1 resulted in increase in mtDNA methylation, DNMT1 might have a role in methylating mtDNA.

Nuclear DNA methylation controls gene expression but very few studies have correlated mtDNA-encoded gene expression levels with changes in mtDNA methylation (Shock, Thakkar et al. 2011, Feng, Xiong et al. 2012, Pirola, Gianotti et al. 2013). As mtDNA methylation was found to be altered by both our experimental approaches, we determined if there were any

associated changes in mtDNA gene expression. Overexpression of DNMT1 led to an overall decrease in transcription of mtDNA-encoded genes, whereas 5-aza-dC increased the expression of most of the mtDNA-encoded genes. Although alteration in mtDNA methylation inversely corresponded with expression of mtDNA-encoded genes, mitochondrial oxygen consumption rate were found to be decreased significantly after both the experimental interventions.

5.2.3 SUMMARY OF FINDINGS: CHAPTER IV – AGING-ASSOCIATED BIOENERGETICS, ITS REGULATORS AND DNA METHYLATION IN A CYTOPLASMIC HYBRID MODEL CREATED FROM PLATELETS OF YOUNG AND OLD HUMAN SUBJECTS

To investigate the direct role of mtDNA in aging-associated mitochondrial dysfunction, cybrids were created from platelets of healthy young and elderly donors; various bio-energetic parameters, factors that control mitochondrial biogenesis and multiple aspects of DNA methylation were studied.

As the nuclear background was kept constant, the only difference across each cybrid was the source of mtDNA in the cybrid model. Our data suggests that mtDNA has a direct role in some of the aging-associated parameters of mitochondrial dysfunction as we found significant reduction in total oxygen consumption rate, basal mitochondrial oxygen consumption rate and complex IV activity (in the isolated mitochondria and whole-cell preparations) in the cybrids generated from elderly donors compared to young donors.

However, no alteration in certain age-dependent bio-energetic parameters such as energy levels (ATP, ADP: ATP ratio) and redox states (levels of NAD^+ and NADH , NAD^+ : NADH ratio) were

found. Paradoxically, we found that complex I activity was significantly elevated in cybrids that were generated from elderly donors. These results suggest the possibility of predominant control by nuclear genome over these bioenergetic parameters, which failed to alter in an age-dependent manner in our model.

Additionally, we found significantly higher mRNA levels of the transcriptional coactivator PRC, transcription factors - NRF1, NRF2 and TFAM (which are known to promote mitochondrial biogenesis) and mtDNA genes coding for Complex I – MTND1 and MTND5 in the cybrids created from aged donors. These results suggest the possibility of nuclear genome- driven compensatory mechanisms in the cybrids created from aged donors, which is further supported by paradoxical increase in complex I activity in cybrids generated from elderly donors.

As we found differences in gene expression levels of both nuclear and mtDNA-encoded genes, multiple aspects of DNA methylation were studied. Although no alteration was found in total DNA methylation, significant increase in mtDNA methylation, mtDNMT1 mRNA levels and DNMT1 protein levels were found in the cybrids made from elderly donors. These results suggest that mtDNA methylation, mtDNMT1 and cellular bioenergetics could be related in an age-dependent manner supporting the rationale to explore the field of mitochondrial-epigenetics.

5.3 STRENGTHS, LIMITATIONS AND FUTURE DIRECTIONS

5.3.1. STUDYING GLOBAL LEVELS OF MTDNA METHYLATION

For approaches that have currently been used for studying global methylation in mtDNA, the presence of nuclear DNA contamination in preparations of mtDNA has not been critically addressed yet. Most of these studies have not characterized purity comprehensively or

considered isolating pure mitochondria (van der Wijst and Rots 2015). We have addressed these issues by isolating gradient purified-mtDNA and then characterizing purity of our mtDNA preparations using qPCR-based methods. Although we found only 0.88% of nuclear DNA remaining in gradient purified mitochondria w.r.t. whole cell DNA, this can be substantial relative to the amount of mtDNA as mitochondrial DNA comprises only a small fraction (less than 1%) of total cellular DNA (because of its very small size, in spite of being present in numerous copies). The estimated percentages of mtDNA were found to be 0.6%, 21% and 41% for whole-cell DNA, crude mtDNA and gradient-purified mtDNA, respectively after accounting for difference in size of nuclear genome compared to mitochondrial genome and also accounting for the mitochondrial DNA copy number. Thus, the fraction of nuclear DNA contamination was estimated to be substantial relative to the mtDNA present in purified mitochondrial preparations and caution must be placed while interpreting our results. However, regression analysis between percentage of mtDNA present in a DNA sample (with increasing levels of mtDNA in DNA extracted from crude and gradient-purified mitochondria w.r.t. whole-cell DNA) and the corresponding percentage methylation, suggested that mtDNA methylation does contribute significantly to overall methylation and mtDNA methylation occurs at least at levels similar to methylation for nuclear DNA. Further in our cybrid studies, where we found a significant increase solely in the DNA methylation from mitochondrial preparations but not from whole cell upon aging, also suggest the presence of mtDNA methylation at significant levels which was not masked by the extent of nuclear DNA contamination. Although our results suggested significant presence of mtDNA methylation at global levels, additional studies will need to be conducted in future to achieve more purified mtDNA in order to substantiate our finding.

Lack of methods to isolate completely pure mitochondrial DNA is a bottleneck in the field of mitochondrial DNA methylation (van der Wijst and Rots 2015). A recent study (Jayaprakash, Benson et al. 2015) established a method to isolate mtDNA from whole-cell DNA using repetitive treatments with Exonuclease V to achieve 98% pure mtDNA. As Exonuclease V selectively degrades single stranded DNA in ATP-dependent manner, the rationale was to eliminate single stranded DNA fragments most likely derived from the nuclear genome, while keeping the circular mtDNA intact. Along similar lines, we attempted purifying mtDNA using repetitive treatments with Exonuclease V followed by column purification. In order to achieve higher purity, we used DNA extracted from isolated mitochondria for Exonuclease V-treatment, instead of just using whole cell DNA, as used in the above mentioned study. Our preliminary data (n =1) suggested unprecedented enrichment of about 1300 fold for mtDNA (compared to 115 fold enrichment in gradient purified mtDNA) using this combinatorial approach of mitochondrial isolation and Exonuclease V treatment. It should be mentioned that crude mitochondrial isolation technique was utilized for this study; thus, we speculate that using gradient purification of mitochondria along with Exonuclease V treatment can further improve the purity of mtDNA preparation. Further studies will need to be conducted in the future in order to examine the reproducibility of this approach followed by analysis of methylation level both at global levels using our established HPLC-MS assay and at specific site using methylation-specific PCR assays.

Further, for our purity characterization using qPCR-based methods, an underlying assumption was that the analysis of tested nuclear and mtDNA-encoded genes (which is miniscule in size compared to whole genome) is representative of overall genomic contamination, which needs to be considered for interpretation of our results. In future, deep-next generation sequencing of

mtDNA-samples needs to be performed to determine the absolute levels of nuclear and mitochondrial DNA present at whole genomic level, for a more accurate analysis.

5.3.2 STUDYING SITE-SPECIFIC LEVELS OF MTDNA METHYLATION

Apart from studying mtDNA methylation at global levels using HPLC MS/MS, the presence of methylation in SH-SY5Y mtDNA was further substantiated using methylation-specific PCR for a specific site in 12S rRNA. We found similar trends of alterations in methylation using both the experimental approaches (oDNMT1 and 5-aza-dC treatment) at both global levels as well as site-specific levels supporting our interpretations. Although, in our study we have used long PCR of mtDNA (an un-methylated amplicon of mtDNA) as negative control, the possibility of obtaining methylation from insertions of mitochondrial DNA in the nuclear genome (NUMT) needs to be ruled out. One of the ways to address this is by utilization of ρ^0 cell lines that lack functional mtDNA, as a negative control in addition to un-methylated amplicon of mtDNA. In a preliminary study, our methylation-specific primers amplified a product from SH-SY5Y ρ^0 cell line raising potential concern about the specificity of primers for mitochondrial genome. However, this analysis requires further confirmatory studies as amplification was also observed from un-methylated amplicon of mtDNA in this study (which was not observed in any of our previous studies) raising concern about possible reagent contamination that needs to be resolved and carefully examined in future. Further, this analysis can also be confounded by the fact that, although ρ^0 cell lines cannot form a functional respiratory chain, but they might have fragments of mtDNA from damaged mtDNA. Alternatively, to address specificity of methylation-specific primers, BLAST analysis was performed which revealed 100% match of primers only to the mitochondrial sequence. However, two nuclear hits in chromosome 11 and 17 were also

identified, but with less than 85% match and mismatches being in the reverse primer sequence suggesting less likely amplification from these sites. Further, it should be noted that the site-specific analysis was conducted using isolated mitochondria where majority of nuclear DNA contamination is eliminated. In contrast to analysis of nuclear contamination at global levels for mtDNA methylation, where small residual nuclear DNA can be substantial compared to mtDNA due to difference in the size of two genomes; for site-specific analysis using methylation specific primer, only two potential sites of NUMT exist for studied region, while copy number of mitochondrial 12S rRNA is much higher (in thousands). Although all these data suggests higher possibility of the amplification being obtained from SH-SY5Y mtDNA instead of the nuclear pseudogenes, further confirmatory studies should be conducted by sequencing the products after methylation specific PCR and then comparing the sequence to mitochondrial genome and potential pseudogene sites and also by following up studies conducted using SH-SY5Y ρ^0 cell line.

In all the research work for this dissertation, we have determined global levels of mtDNA methylation using HPLC-ESI-MS/MS and mtDNA methylation at a particular site in 12S rRNA using methylation-specific PCR assay. Hence, we are not aware of the status of modified cytosines at other specific sites in the mtDNA genome, which can be addressed by bisulfite- next generation sequencing. One of the future experiments will be to determine the specific sites where mtDNA modifications have occurred after both the experimental interventions (i.e. DNMT1 overexpression and 5-aza-dC treatment) and in the cybrid model using bisulfite-next generation sequencing and methylation-specific PCR for other regions in mtDNA. This will provide us an in-depth view of the altered sites of methylation in mtDNA. One of the limitations of bisulfite-based methods that are used to study site-specific mtDNA methylation is that the

modified cytosines such as 5mC and 5hmC cannot be distinguished. Single molecule Real Time (SMRT) sequencing can also be used for future investigations to resolve this issue. SMRT sequencing can distinguish between modified cytosines due a difference in the time of incorporation of cytosine during synthesis for sequencing (Davis, Chao et al. 2013, Chavez, Huang et al. 2014, Yu, Ji et al. 2015).

5.3.3 MECHANISM AND FUNCTIONAL RELEVANCE OF MTDNA METHYLATION

In chapter III, a limitation of our experimental approach is that the overexpression of DNMT1 and 5-aza-dC treatment could have affected the methylation levels in both the nuclear as well as the mitochondrial genome. As mitochondrial transcription is also regulated by nuclear-encoded genes, thus, the effects observed on mtDNA transcription by overexpression of DNMT1 and 5-aza-dC treatment can be indirect consequence of alteration in nuclear DNA methylation and transcription. Interestingly, the mRNA levels of TFAM were not altered by both the experimental approaches and TFAM being the major nuclear-encoded regulator of mitochondrial transcription suggests that there is a possibility that it's the differences in mtDNA methylation which are responsible for changes in transcription of mtDNA-encoded genes. However, the causal role of altered mtDNA methylation exclusively in affecting mitochondrial transcription cannot be confirmed definitively and needs to be addressed in future. Nonetheless, how mtDNA methylation can be selectively altered is not yet known, which has also hindered the field from pursuing mechanistic studies on mtDNA methylation. Irrespective of this limitation, ours is the first study to demonstrate that mtDNA methylation can be altered experimentally, which was previously not known. Further, we are the first to demonstrate that changing DNMT1 levels alters mtDNA methylation. Furthermore, previous studies only observed changes in mtDNA

methylation in bio-specimens from various pathological conditions and a few of these studies concomitantly observed changes in transcription of some of the mtDNA-encoded genes (Shock, Thakkar et al. 2011, Feng, Xiong et al. 2012, Pirola, Gianotti et al. 2013). The arena of functional significance of mtDNA methylation in the context of regulating mitochondrial transcription and function was completely unexplored. Our study provided the first significant experimental evidence in the direction that alteration of mtDNA methylation can associate with mitochondrial transcription and function. This will provide a platform for future studies in this direction to get more valuable insights on the functional relevance of mtDNA methylation.

Although our work provided novel insights on several aspects of mtDNA methylation, our studies did not investigate mechanisms how mtDNA methylation can be affecting mitochondrial transcription and function. Future studies focused on site-specific methylation in mtDNA and studies investigating effects of alteration of mtDNA methylation at specific sites on binding of different proteins (that comprise the transcription machinery - TFAM, POLRMT and TFB2M) to mtDNA, especially in the D-loop region, will allow us to investigate how these sites of mtDNA methylation are linked to altered mitochondrial transcription. What determines synthesis of mitochondrial isoform of DNMT1 along with triggering its translocation to mitochondria needs to be studied. A histone-like role of TFAM is emerging, and few studies also reported that post-translational modifications of TFAM can affect compaction or loosening of mtDNA and ultimately affecting transcription of mtDNA-encoded genes. Thus, an intriguing possibility is that mtDNA methylation may be regulating post-translational modifications of TFAM and thus affecting transcription by mtDNA methylation, which will be studied in future. All of these experiments might provide some mechanistic insight into how the transcription of mtDNA gets altered after experimental manipulations used in this study.

In chapter IV, we have found an age-dependent alteration in mtDNA methylation in the cybrid model. However, further studies are required to delineate role of mtDNA methylation as a cause or an effect in age-associated mitochondrial dysfunction. Further, experiments need to be carried out in order to reverse the age-dependent alteration in mtDNA methylation in the cybrid model, and investigate if this reversal in mtDNA methylation altered mitochondrial function. But direct role of mtDNA methylation in aging-associated mitochondrial dysfunction will be difficult to establish, considering that how to exclusively alter mtDNA methylation is still not known.

5.4 CONCLUSION

The purpose of this dissertation was to establish a method to quantitate mtDNA methylation globally and evaluate the presence of mtDNA methylation in SH-SY5Y neuroblastoma cells; further, to examine whether mtDNA methylation can be altered experimentally and also if there are any associated changes in mitochondrial transcription and function; and finally, to investigate if mtDNA plays a role in mitochondrial dysfunction during aging using cybrid model and if mtDNA methylation is also altered in this model.

We have established a direct method to quantify global methylation in mtDNA using HPLC-ESI-MS/MS along with addressing issues of variable DNA digestion and the possible chances of nuclear DNA contamination. Also, we have found that global mtDNA methylation occurs at a level of 3% in SH-SY5Y gradient-purified mtDNA. Existence of mtDNA methylation was also confirmed by studying methylation at a particular site (12S rRNA) in mtDNA. Further, we found that mtDNA methylation is an alterable phenomenon, which can be influenced by levels of DNMT1 and treatment with DNA methylation inhibitor (5-aza-dC). Overexpression of DNMT1 increased whereas 5-aza-dC treatment decreased mtDNA methylation, with concomitant inverse

changes in mtDNA transcription. Our data suggests that alterations in mtDNA methylation are associated with changes in expression of mtDNA-encoded genes and mitochondrial function. Finally, using cybrid model created from platelets of young and aged donors, we established a direct role of mtDNA in aging-associated mitochondrial dysfunction and found mtDNA methylation to be altered in an age-dependent manner.

CHAPTER VI: REFERENCES

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